K_ATP channels and preconditioning: A re-examination of the role of mitochondrial K_ATP channels and an overview of alternative mechanisms

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Abstract

Preconditioning by one or several brief periods of ischemia activates an endogenous cardioprotective program that increases the resistance of cardiomyocytes to injury by subsequent prolonged periods of ischemia. Ischemic preconditioning can be mimicked by K⁺ channel openers and various other substances, a phenomenon termed pharmacological preconditioning. Initially, ischemic preconditioning has been ascribed to the opening of ATP-sensitive K⁺ channels at the surface membrane of cardiomyocytes. Since 1997, numerous publications have implicated mitochondrial ATP-sensitive K⁺ channels (mK_ATP) as a major trigger and/or end effector of preconditioning. Diazoxide has been suggested to be a specific activator of mK_ATP channels, and the substituted fatty acid 5-hydroxydecanoate (5-HD) has been suggested to be a specific inhibitor. However, diazoxide and 5-HD have multiple K⁺-channel-independent actions, and the experimental evidence for an obligatory role of mK_ATP channels in preconditioning, or even their existence, remains inconclusive. In contrast, surface K_ATP channels have been well characterized, and we summarize the evidence suggesting that they make a major contribution to preconditioning. We also discuss a number of other factors involved in preconditioning: (1) generation of reactive oxygen species, (2) impairment of fatty acid metabolism, and (3) opening of the mitochondrial permeability transition pore. In the light of these emerging concepts, we critically re-examine the evidence for and against a role of mK_ATP channels in ischemic and pharmacological preconditioning.

Keywords: Diazoxide; 5-Hydroxydecanoate; K_ATP channels; Ischemic preconditioning

1. Introduction

Preconditioning activates a powerful endogenous adaptive mechanism that increases the resistance of the heart to ischemia and could potentially increase the chances of survival under pathophysiological conditions. Two different types of ATP-sensitive potassium channels have been implicated in preconditioning: surface membrane K_ATP (sK_ATP) channels and mitochondrial K_ATP (mK_ATP) channels. The structure and function of sK_ATP channels have been investigated extensively, whereas the subunit composition and the gene(s) coding for putative mK_ATP channels are still unknown. Both channels are presumed to be regulated by changes in energy metabolism and to have cardioprotective effects.

The central question of this review is whether mK_ATP, sK_ATP or both of these channels play a role in ischemic and/or pharmacological preconditioning. We first provide an overview of the phenomenon of preconditioning and outline the concept of mK_ATP channels as a major mediator of preconditioning (Section 2). Despite a large body of work on intact hearts and on simplified models of preconditioning, doubts persist as to the plausibility of current hypotheses about mK_ATP channels as triggers and/or effectors of preconditioning. By nature of their localization, mK_ATP channels are difficult to study. We therefore discuss the experimental evidence for the presence of mK_ATP channels in the mitochondrial inner membrane in some detail and point out the merits and pitfalls of the different methodological approaches (Section 3).

The main tools for discriminating between mK_ATP channels and sK_ATP channels are the K⁺ channel openers diazoxide, nicorandil and pinacidil and the blockers 5-hydroxy-
decanoate (5-HD), HMR1098 and glibenclamide. However, several recent studies have questioned the usefulness of these substances for defining and characterizing mKATP channels. To assess the validity and limitations of the pharmacological approach to channel characterization, we provide an analysis of the effects of diazoxide and 5-HD on mKATP channels and on other targets (Section 4). On the basis of the available data we summarize our view of the role of mKATP channels in preconditioning. In addition, we discuss the possible role of other mitochondrial K⁺-selective channels in regulating matrix volume and the potential of the mitochondrial inner membrane (Section 5).

The surface KATP channel is a sensor of the metabolic state of the cells that is modulated by a variety of metabolic intermediates. It is clear that it does contribute to the effects of preconditioning, but the extent of its contribution is still under debate. We discuss the mechanisms of regulation of the sKATP channel that may be relevant under conditions of ischemia, and we summarize the pharmacology of sKATP channels, emphasizing similarities and differences between sKATP and mKATP channels (Section 6). We then go on to summarize the information available about the role of sKATP channels in preconditioning (Section 7). Finally, we give an overview of the most important alternative mechanisms of preconditioning. In particular, the profound alterations of fatty acid metabolism occurring during ischemia and reperfusion, as well as the regulation of the mitochondrial permeability transition pore by reactive oxygen species may play a role as mediators or effectors of cardioprotection.

The intention of this review is not to say that the current view of the mKATP channel as the principal mediator of preconditioning is necessarily incorrect, or that mKATP channels do not exist. In fact, there is ample evidence for the existence of K⁺ channels in the mitochondrial inner membrane, and there is growing consensus that ion channels are involved in the regulation of matrix volume, matrix calcium and respiratory rate. Rather, we want to point out that the properties ascribed to mKATP channels on the basis of pharmacological experiments should not be considered as established facts. It is not yet clear whether the mitochondrial inner membrane is endowed with channels that bear any resemblance to surface KATP channels, and alternative hypotheses for the cellular defense mechanisms against ischemic damage also need to be considered. In the summary and conclusions of this review we try to weigh the evidence for and against a role of mKATP and sKATP channels in preconditioning in the light of recent findings, and we try to give an integrated picture of the mechanisms involved in protecting the heart against ischemic injury.

2. Studies of preconditioning in the intact heart

2.1. The phenomenon of preconditioning

The term ischemic preconditioning (IPC) refers to the observation that one or several intermittent periods of ischemia (lasting ~ 5 min) protect the myocardium against the injury caused by a subsequent, prolonged period of ischemia (lasting ~ 30 min), which is denoted index ischemia [1–4]. There are two windows of protection: the initial window which lasts 1–2 h after the preconditioning stimulus (“classical IPC”) and the ‘second window of protection’ which is less effective but covers a longer period. It occurs about 24 h after the preconditioning stimulus and lasts about 72 h. The possible mechanisms underlying the second window of protection have been reviewed elsewhere [3,5,6] and will not be discussed here. The cardioprotective effect of brief periods of ischemia can be mimicked by exposing the heart to various drugs, a phenomenon termed pharmacological preconditioning (PPC). The drugs used for PPC include K⁺ channel openers (diazoxide, pinacidil and nicorandil), volatile anesthetics (halothane, desflurane, isoflurane and sevoflurane) [7], various agonists of G protein-coupled receptors (adenosine, bradykinin, catecholamines and opioids) [6,8–13], succinate dehydrogenase blockers (3-nitropropionic acid) [14], carbonmonoxide releasing molecules [15] and Na⁺/H⁺ exchange blockers (cariporide and ethylisopropyl amiloride) [16]. In most cases, PPC produces a similar degree of protection against subsequent ischemic injury as IPC.

It is generally assumed that preconditioning activates endogenous intracellular defense mechanisms that increase the tolerance to injury [3,6]. These endogenous cell survival programs are probably highly complex and depend, among other factors, on mitochondrial and cytosolic energy metabolism and the electrical activity of the cells [3,17,18]. A schematic description of the protection afforded by IPC and PPC is given in Fig. 1, which shows that the activation of cardioprotective mechanisms does not prevent, but rather delays cellular death. When the optimal preconditioning stimulus (brief episodes of ischemia or application of cardioprotective drugs) and the optimal duration of the index ischemia (usually 30–40 min) are chosen, infarct size after reperfusion is substantially reduced as compared to control, i.e. the infarct size measured without preconditioning. However, with shorter or longer duration of the index ischemia the difference between hearts with and without preconditioning stimulus is much less conspicuous. In other words, the effectiveness of preconditioning (reduction of infarct size compared to control) depends critically on the duration of the index ischemia chosen. Furthermore, the effectiveness of preconditioning depends on the timing of the index ischemia. When the interval between the conditioning stimulus and the onset of the index ischemia is postponed, the cardioprotective effect becomes smaller and eventually disappears within 2 h, indicating the time course of the decay of the defense mechanisms involved in IPC.

It is likely that preconditioning also occurs in human heart [3,6,19], and the elucidation of the mechanisms underlying the increased tolerance to ischemia is of considerable clinical importance [3,20–24]. Although the most extensive studies of preconditioning have been carried out in the heart, similar protective phenomena induced by short periods of ischemia have also been reported for the brain, skeletal muscle [25–27], kidney [28] and liver [29,30].
2.2. The receptors and intracellular messengers involved in preconditioning

Most studies of preconditioning were carried out using occlusion of the left coronary artery or its branches in canine [31], porcine [32], rabbit [33–37] or rat [38–40] hearts. This experimental model has the advantage that it mimics the ischemic events in patients suffering from acute coronary syndromes. The readout of the cardioprotective effect is usually infarct size, expressed as percentage of the risk zone. Reduction of infarct size to approximately 25% of control (see Fig. 1), or to even smaller values, has been reported by many laboratories [3,41]. A disadvantage, though, of the in vivo approach is that the animals are (necessarily) anesthetized by agents which by themselves may modify infarct size following prolonged ischemia. In fact, inhalational anesthetics [13,42–44], as well as opioid anesthetics [11,45], are cardioprotective whereas various intravenous anesthetics such as ketamine, thiopental and pentobarbital have been shown to inhibit pharmacological preconditioning [46]. Hence, the choice of anesthetic for preconditioning studies in vivo, which is usually ketamine, thiopental and/or pentobarbital, may profoundly affect the measured end point (infarct size), as demonstrated by Cope et al. [42].

After nearly 20 years of intensive investigation a partial picture of the signal transduction pathways involved in preconditioning has emerged (Fig. 2). Various agonists that are known to accumulate in the interstitium during ischemia, such as adenosine, bradykinin, norepinephrine and opioids, have been shown to confer cardioprotection [33,47]. Many of the “cardioprotective” agonists couple to phospholipase C (PLC) [48,49]. Activation of PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP$_2$), generating the secondary messengers inositol 1,3,4-trisphosphate (IP$_3$) and diacylglycerol (DAG), the latter stimulating protein kinase C (PKC) (Fig. 2). There is convincing evidence that PKC is a central mediator of preconditioning [34,50–53]. Liu et al. [54] even proposed that activation of PKC, which requires its translocation from the cytosol to anchoring proteins on the membrane cytoskeleton [55], produces the preconditioned state. This idea was supported by experiments showing that PKC activators mimic the effects of IPC [56–59]. PKC may directly phosphorylate the effector proteins of preconditioning [56–59]. PKC may directly phosphorylate the effector proteins of preconditioning (Fig. 2) or switch on kinase cascades such as mitogen activated protein kinases [60]. Although, the role of PKC as a trigger of preconditioning is not undisputed [3,60], it has been emphasized that specific isoforms of PKC may behave quite differently [49]. A good case, though, can be made for PKCε since this isoform translocates from the cytosol to the surface membrane and to mitochondria during ischemia [61–68].

Activation of protein kinase a (PKA), independent of PKC, has also been shown to be cardioprotective, possibly through the inhibition of Rho-kinase [69]. Consistent with a role for PKA, β-adrenergic receptor agonists and increased cAMP have been shown to protect the heart from ischemia [70]. Hence, both the β-adrenergic/cAMP and PLC-linked signaling pathways, the latter of which includes α$_1$-adrenergic receptors [11], can independently, and possibly synergistically, confer protection from ischemia. Other recent studies on the mechanisms of ischemic and pharmacological preconditioning...
ing have implicated mitogen activated protein kinases (MAPKs) [60,71–76] and phosphatidylinositol-3-kinase (PI3K) [77–80].

Reactive oxygen species (ROS), which are known to activate various kinases including PKC, also play a role in IPC and PPC. Baines et al. [81] showed that a free radical scavenger could block preconditioning induced by one or more short ischemic episodes. Further work in Downey’s laboratory revealed that the receptor agonists acetylcholine, bradykinin, opioids and phenylephrine, but not adenosine, triggered preconditioning by generating ROS [11]. PPC with the K⁺ channel openers diazoxide and pinacidil [82] and with the anesthetic agents halothane, isoflurane and sevoflurane [83–87] has also been shown to increase ROS in isolated myocytes. Moreover, the protective effects of these drugs were blocked by antioxidants. Hence, pharmacological and ischemic preconditioning probably share generation of ROS as a common signaling pathway. The downstream targets of ROS have not been fully elucidated, but probably include PKC [88,89] (Fig. 2) and other kinases [90–93].

The search for triggers/mediators of preconditioning turned a new corner after the publication of seminal papers by Garlid et al. [94] and Liu et al. [95], who proposed that ischemic preconditioning may be related to the opening of mitochondrial K<sub>ATP</sub> channels. Liu et al. carried out measurements of flavoprotein fluorescence in intact rat ventricular cardiomyocytes, and Garlid et al. partially purified mK<sub>ATP</sub> channels and characterized their pharmacology [94,96–98] [99]. On the basis of these measurements (see Sections 3.2, 3.3 and 3.4) it was proposed that mK<sub>ATP</sub> channels play an obligatory and central role in preconditioning. Moreover, it was postulated that diazoxide selectively opens, and 5-HD specifically blocks, mK<sub>ATP</sub> channels. Numerous subsequent publications were based on the assumption that experimental observations that can be mimicked by diazoxide and blocked by 5-HD are attributable to the opening of mK<sub>ATP</sub> channels (reviewed in Refs. [1–4,100–102]). However, no consensus has been reached whether the opening of mK<sub>ATP</sub> channels acts as a trigger of the cardioprotective effect (opening during the triggering phase) or as an end effector (opening during or after the index ischemia) [3,31,37,67,103–105]. In most earlier studies mK<sub>ATP</sub> channels were proposed to be end effectors, and the channels were assumed to open during the index ischemia [31,106–108]. In some recent studies, opening of mK<sub>ATP</sub> channels has been inferred to be the initial trigger of the cardioprotective effect, promoting the generation of ROS and inducing the activation of PKCe [37,82,91,103], whereas Lebuffe et al. [109] proposed that generation of ROS during the trigger phase leads to the opening of mK<sub>ATP</sub> channels. Some authors have suggested that mK<sub>ATP</sub> channels act both as a trigger and as an end effector of IPC [1,3].

In conclusion, many groups have reported that diazoxide mimicked and 5-HD prevented preconditioning. In fact, the effects of these drugs on infarct size have become something like the gold standard for testing whether a phenomenon is due to opening of mK<sub>ATP</sub> channels. For a detailed coverage of these experimental findings, and their potential shortcomings, the reader is referred to comprehensive recent reviews.

Fig. 2. Schematic diagram of some of the mechanisms thought to be involved in the activation of mK<sub>ATP</sub> and sK<sub>ATP</sub> channels. The targets of diazoxide, glibenclamide and 5-HD are indicated. Abbreviations: R, receptor; G, G-protein; PLC, phospholipase C, PKC, protein kinase C; ETC, electron transport chain.
Nevertheless, some doubt about the mKATP channel hypothesis remained. Since it turned out to be difficult to pin down the mechanisms involved in IPC and PPC using solely studies of infant size, more reductionist models of preconditioning have been introduced, for instance, application of global ischemia and monitoring post-ischemic recovery in isolated hearts [110–113], monitoring changes in intracellular calcium, electrical activity and morphology in isolated cardiomyocytes [114–121], or studying the behavior of mitochondria in intact cardiomyocytes [122,123]. These simpler experimental models could indeed reproduce some of the essential features of IPC and PPC, and this approach turned out to be of great value for identifying discrete components of the endogenous defense program of cardiomyocytes (see Sections 7 and 8). However, in some of the recent studies, the assumption that preconditioning is mediated by mKATP channels has been questioned. Since most of the information on the pharmacology and regulation of mKATP channels has in fact been derived from studies of preconditioning, we re-examine the more direct evidence for the existence of mKATP channels (independent of preconditioning) in the next section.

3. The search for mitochondrial KATP channels

3.1. Single-channel studies in mitoplasts

The first report suggesting the existence of mKATP channels was published in 1991 by Inoue et al. [124], who measured ATP-sensitive K+ currents with a conductance of ~10 pS in inside-out patches excised from fused giant mitoplasts. One concern with this experimental approach is that contaminating surface membrane could have introduced KATP channels to the mitoplasts (mitochondria chemically divested of their outer membrane, ruptured and reformed). The standard procedures used to isolate mitochondria, which involve tissue homogenization, provide ample opportunity for the cross-coalescence of sarcolemmal, endoplasmic reticular and mitochondrial membranes, and Inoue et al. [124] did not employ membrane markers to quantify the extent of contamination of the mitoplasts with sarcolemmal proteins. Another concern is that the patches contained many different channels, and that, apparently, inhibition of the putative KATP channel was only observed after application of ~0.5 mM ATP. Moreover, when more than one channel was active in the patch the open-state was found to be unstable, which was interpreted to indicate ‘cooperative transitions between dual or multi states’ [124]. The authors also reported that Mg2+ was not necessary for ATP-induced inhibition and ADP (2 mM) had no effect. In many aspects, this study was quite preliminary and its methodological limitations do not allow any reliable inferences on the nature of mitochondrial K+ channels.

In another study, mitoplasts prepared from a human T-lymphocyte cell line were investigated to detect mKATP channels [125]. A channel with a conductance of ~80 pS at positive potentials was seen in 12 out of 260 patches. It showed strong outward rectification and was apparently not K+ selective. Only a minor decrease in open probability was observed upon application of 0.5 or 6 mM ATP to the matrix side of the patch, which was irreversible and might therefore represent rundown of the channel. At a concentration of 25 mM, ATP had no effect. Application of 1 mM 5-HD caused a slight, irreversible, time-dependent reduction in open probability, which may also have been due to rundown. Diazoxide had no significant effect. Thus, there is actually no good reason to denote this channel as a mKATP channel.

Er et al. [126] prepared fluorescent mitoplasts from rat cardiomyocytes preloaded with TMRE. The mitoplasts apparently had no membrane potential. In “mitoplast-attached” measurements they observed a channel with a linear current–voltage relation and a conductance of ~13 pS. The channel had very slow kinetics and a very low open probability, which could be increased after application of diazoxide (100 µM) or testosterone (10 µM). The K+ selectivity of the channel was not studied. The authors argued that the channel is blocked by ATP from the matrix side and has a very low open probability even in the absence of any blockers on the cytosolic side. This contradicts the conclusions obtained with reconstituted mKATP channels in proteoliposomes [101,127] (see Section 3.2). It should also be noted, that retention of TMRE in the isolated mitochondrial preparation does not necessarily indicate that the mitochondria remained intact during the isolation procedure since the dye, in addition to electrophotoretic accumulation, can bind to the inner membrane [128].

Recently, evidence for expression of a voltage-activated K+ channel (Kv1.3), with a conductance of about 25 pS [129], and of a Ca2+-activated K+ channel [4,130] in the mitochondrial inner membrane has been obtained. Another mitochondrial ion channel, the classical 107 pS channel [131], is also permeable to K+ ions. In conclusion, although several mitochondrial K+ permeable channels have been described, there is, as yet, no convincing electrophysiological evidence for a native K+-selective channel in the mitochondrial inner membrane that has a high sensitivity to adenine nucleotides, diazoxide and 5-HD.

3.2. Reconstitution of mKATP channels in liposomes and lipid bilayers

Another source of evidence for the existence of mKATP channels came from the work of Paucek et al. [96]. A protein fraction containing a K+ channel was extracted from detergent-solubilized membranes of isolated rat liver and beef-heart mitochondria and reconstituted into liposomes or planar lipid bilayers. The dominant protein in the purified fraction had a molecular mass of 54 kDa. As in the case of the mitoplast studies [124–126], Paucek et al. [96] did not provide evidence to rule out that the extract could have been contaminated with surface membrane proteins. Interestingly, the molecular mass of the reconstituted protein [96] would fit the
mitochondrial Ca\(^{2+}\)-activated K\(^+\) channel in cardiac muscle (\(M_{\text{mt}}\), ~55 kDa) [132], or the adenine nucleotide translocase (ANT; \(M_{\text{mt}}\), ~30 kDa), which exists as a dimer [133].

Using liposomes containing the reconstituted 54 kDa protein and loaded with the fluorescent K\(^+\) indicator PBFI, Paucek et al. [96] tried to assess changes in the open probability of mK\(_{\text{ATP}}\) channels by measuring CCCP-dependent ‘electrophoretic K\(^+\) uptake’. They found that K\(^+\) uptake into the proteoliposomes was inhibited by ‘cytosolic’ ATP or ADP, but only when either Mg\(^{2+}\) or Ca\(^{2+}\) was present. To explain the apparent discrepancy with the findings of Inoue et al. [124] it was suggested [96] that the solutions used by Inoue et al. were not really Ca\(^{2+}\)-free but contained about 100 nM free Ca\(^{2+}\). The same argument could be applied to the conflicting data of Er et al. [126] since these authors did not include a Ca\(^{2+}\)-chelator in their nominally Ca\(^{2+}\)-free solutions. Surprisingly, the inferred K\(^+\) flux into the liposomes was insensitive to tetraethylammonium (TEA) ions [96], which would clearly distinguish the putative mK\(_{\text{ATP}}\) channel from sK\(_{\text{ATP}}\) channels [134] and other K\(^+\) channels [135]. The inferred K\(^+\) influx was inhibited by glibenclamide with an IC\(_{50}\) value of ~50 nM [96]. In contrast, high concentrations of glibenclamide (>5 μM) were apparently required to inhibit the ATP-sensitive ion channel observed electrophysiologically [124,126].

Zhang et al. [136] investigated the effects of MgATP, diazoxide, 5-HD, glibenclamide and ROS on ion channels reconstituted from submitochondrial membrane vesicles and incorporated into planar lipid bilayers. Application of 1 mM MgATP to the trans side of the bilayer (which corresponds to the matrix side) inhibited the channel significantly, but MgATP had no effect on the cis (cytosolic) side, in contrast to the conclusions drawn by Garlid and co-workers [127]. The effects of Mg\(^{2+}\) or ATP alone were apparently not tested. With symmetrical 150 mM K\(^+\) the channels had a unitary conductance of 56 pS, which is much higher than the value reported for the mK\(_{\text{ATP}}\) channel in heart and liver mitochondria (10–15 pS [124,126]). The open-state probability of the channel was reduced by 5-HD (100 μM) and glibenclamide (50 μM), whereas diazoxide and ROS increased channel activity [136]. The selective sK\(_{\text{ATP}}\) channel blocker HMR1098, used to exclude sarcolemmal channels, had no effect.

More recently, Ardehali et al. [137] tried to reproduce some aspects of earlier work [96] on reconstituted mK\(_{\text{ATP}}\) channels in proteoliposomes. They suggested that their mK\(_{\text{ATP}}\) channel, partially purified from a fraction of rat mitochondrial inner membrane, may consist of a complex of at least five proteins including succinate dehydrogenase (SDH) and ANT [137]. The reconstituted ‘mK\(_{\text{ATP}}\)’ channel was now described as a non-selective cation channel [137], and the lack of K\(^+\) selectivity was attributed to the presence of low concentrations of dithiothreitol. No evidence was presented that the channel was impermeable to anions. The electrophoretic cation influx into liposomes was moderately increased (by a factor of ~2.8) in the presence of 100 μM diazoxide, and this increase could be partially inhibited by 500 μM 5-HD, 10 μM glibenclamide or 2 mM ATP, in the absence of Ca\(^{2+}\) and Mg\(^{2+}\). The cation influx was also moderately increased (by a factor of ~2.3) by the SDH inhibitor 3-nitropropionic acid. When the protein complex was reconstituted in lipid bilayers, a channel with a conductance of 200 pS (with symmetrical 500 mM KCl) was found [137]. The authors interpret their findings to indicate that “SDH regulates mK\(_{\text{ATP}}\) channels by means of its physical interaction with the ionophore”. In comparison, the reconstituted mK\(_{\text{ATP}}\) channel described by Paucek et al. [96] was K\(^+\) selective, showed no inhibition by ATP in the absence of divalent cations and had a conductance of 30 pS (with symmetrical 1 M KCl) [96]. In this and several other aspects, the putative mK\(_{\text{ATP}}\) channels described by the two groups appear to be clearly different.

In conclusion, the mK\(_{\text{ATP}}\) channels reconstituted in proteoliposomes or lipid bilayers by different groups differ in many aspects, such as molecular mass, ion selectivity, glibenclamide sensitivity, inferred orientation in the membrane and conductance. It is likely that the purification procedure of the proteins and the lipid composition of the membrane may have influenced the results. The technical difficulties of inserting native and cloned ion channels in lipid bilayers are well known [138,139]. In addition, validation of the PBFI fluorescence assay in proteoliposomes with a known K\(^+\) channel protein would be desirable to define more clearly the limitations of this method. Further studies are required to define the pore-forming constituents of the five-protein complex containing SDH and ANT [137]. It will be interesting to see if there is any relation of this protein complex to the mitochondrial permeability transition pore (MPTP), which also contains ANT [140–142].

3.3. Measurement of matrix volume in isolated mitochondria

There is general agreement that opening of mitochondrial K\(^+\) channels, accompanied by the movement of permeable anions, should lead to an increase in matrix volume [4,96,101,143–145], and that the increase in matrix volume will activate the respiratory chain [100,143]. Therefore, measurements of matrix volume have played an important role in the investigation of mK\(_{\text{ATP}}\) channels. A widely used technique for determining such volume changes is to monitor the light scattering of mitochondrial suspensions; an increase in mitochondrial volume is associated with a decrease in light scattering. Changes in light scattering have been used as an indirect measurement of K\(^+\) influx, which should depend on the potential of the mitochondrial inner membrane and on the “cytosolic” concentration of K\(^+\) [97,146]. Garlid and co-workers reported that the initial rate of change of light scattering following addition of mitochondria to the cuvette (indicating mitochondrial swelling) was inhibited by ATP, ADP and AMP [99,146]. K\(^+\) channel openers such as diazoxide increased the change of light scattering [97], and this effect was inhibited by the putative mK\(_{\text{ATP}}\) channel blockers glibenclamide and 5-HD [99]. Garlid and co-workers emphasize that these changes in light scattering were not observed...
In conclusion, the experimental data summarized in this section are consistent with the notion [143] that there is a K⁺-dependent regulation of matrix volume. However, the published measurements of changes in matrix volume with diazoxide and 5-HD are contradictory, and the measurements with adenine nucleotides are open to alternative interpretations, swaying versus conformational changes of the ANT. Furthermore, the characterization of the molecular nature of the ion channels responsible for the volume changes hinges on the specificity of the channel openers and blockers used (see Section 4).

3.4. Flavoprotein fluorescence as an indicator of mK<sub>ATP</sub> channel activity

In their pioneering papers on mK<sub>ATP</sub> channels, Marban and co-workers tested the effects of diazoxide and 5-HD on flavoprotein fluorescence in intact rabbit cardiac myocytes [95,98]. The rationale for measuring flavoprotein fluorescence was that “opening of mitoK<sub>ATP</sub> channels dissipates the inner mitochondrial membrane potential established by the proton pump. This dissipation accelerates electron transfer by the respiratory chain and, if uncompensated by increased production of electron donors, leads to net oxidation of the mitochondria” [98] (note that oxidized, but not reduced, flavoproteins are fluorescent). Diazoxide produced a dose-dependent increase in flavoprotein fluorescence, assumed by the authors to signify opening of mK<sub>ATP</sub> channels, which was blocked by 5-HD. In contrast to these data obtained with rabbit ventricular myocytes, diazoxide has been shown to have no effect on flavoprotein fluorescence measured in freshly isolated rat [116] or guinea-pig ventricular myocytes [155].

According to a review by Garlid et al. [2], the majority of myocytes used by Marban’s group for flavoprotein fluorescence experiments were incubated overnight in substrate-free medium. However, Brian O’Rourke (personal communication) asserted that cells were in fact incubated overnight in M199 medium, which contains ample metabolic substrate, whereas experiments were performed in substrate-free modified Tyrode’s solution. Nevertheless, the use of substrate-free solutions complicates the interpretation of the flavoprotein fluorescence experiments, especially since 5-HD is known to be metabolized, albeit poorly, by the β-oxidation pathway of mitochondria [147,155–157], and fatty acid metabolism induces flavoprotein reduction [158]. Thus, metabolism of 5-HD by the substrate-deprived cells could have been responsible for the observed decrease in flavoprotein fluorescence observed after addition of 5-HD. Furthermore, at concentrations similar to that used by Sato et al. [98], diazoxide inhibits succinate dehydrogenase [147,155,159–161], which itself can increase flavoprotein fluorescence [155]. Another problem with the flavoprotein fluorescence assays was that glibenclamide alone had an effect. Liu et al. [95] reported that, “[They] did not observe consistent blockade of mitochondrial oxidation, probably because glibenclamide alone caused oxidation of the flavoproteins especially at concentrations > 1 µmol/l (data not shown).”
Recently, Sato et al. [123] confirmed the oxidation of flavoproteins by diazoxide in intact cardiomyocytes in glucose-free solution. Interestingly, they found that opening of BKCa channels with NS1619 (30 μM) oxidized flavoproteins to about the same extent as diazoxide (100 μM). The effects of NS1619 could be inhibited with paullin, a blocker of BKCa channels. These findings underscore the potential usefulness of flavoprotein fluorescence as an indicator of the rate of electron transfer by the respiratory chain. On the other hand, the discovery of BKCa channels in the mitochondrial inner membrane makes the interpretation of some of the earlier data more complicated because now there is a competing K+ conductance.

### 3.5. Measurement of mitochondrial membrane potential

Measurements of $\Delta \Psi_m$ have been very useful in the investigation of the function of mitochondrial ion channels. Because Ca$^{2+}$ uptake into mitochondria is driven primarily by $\Delta \Psi_m$, small changes in $\Delta \Psi_m$ can induce large changes in matrix Ca$^{2+}$ concentration [123,162], and thus large changes in the functional state of mitochondria. Kowaltowski et al. [145] argued that in fully energized mitochondria the change in $\Delta \Psi_m$ induced by the opening of mKATP channels should be very small, and that the main result of mKATP channel opening in the dynamic steady state is an increase in matrix volume. This is so because the K+ influx is followed immediately by K+/H+ exchange and uptake of phosphate (P_i) via the electroneutral P/OH– exchange carrier. Indeed, Ishida et al. [162] found that 100 μM diazoxide-induced only a small, not significant, depolarization of mitochondria in rat ventricular cardiomyocytes and did not affect matrix Ca$^{2+}$, measured as Rhod-2 fluorescence. However, when cytosolic and matrix Ca$^{2+}$ was increased by inhibiting the Na+,K+-ATPase with ouabain, 100 μM diazoxide did produce a significant depolarization and a decrease in matrix Ca$^{2+}$ concentration [123,162]. Since these data, and from their flavoprotein fluorescence measurements, Sato et al. [123] concluded that opening of either mKATP channels or BKCa channels reduces mitochondrial Ca$^{2+}$ overload by decreasing $\Delta \Psi_m$, in accord with the work of Holmuhamedov et al. [163].

It should be mentioned, though, that alternative interpretations of the effects of diazoxide on $\Delta \Psi_m$ have been put forward. In isolated rat heart mitochondria it was found that 100 μM diazoxide or 100 μM pinacidil substantially accelerated state-4 respiration and decreased $\Delta \Psi_m$ [164]. Since these effects could be mimicked by application of 2,4-dinitrophenol (DNP) and were maintained in K+-free medium the authors concluded that the effects of the two K+ channel openers were mediated by an uncoupling action, in agreement with Kowaltowski et al. [145]. Interestingly, application of DNP, like diazoxide or pinacidil, improved functional recovery of isolated rat hearts from ischemia reperfusion, suggesting that uncoupling per se could be cardioprotective [164–166]. Thus, there may also be a K+-channel-independent pathway for cardioprotection by K+ channel openers, related to a decrease in ROS production induced by uncoupling.

In a series of experiments in which isolated mitochondria were investigated under conditions mimicking ischemia, Korge et al. [154,167,168] demonstrated that regulation of K+ conductance and matrix volume may be critical for cardioprotection. They suggested that when mitochondria are in a weakened state, with reduced transport capacity of the respiratory chain (for example, during ischemia or at the onset of reperfusion), K+ influx and the resulting increase of matrix volume and decrease in $\Delta \Psi_m$ may be specially relevant for avoiding excessive matrix shrinkage, Ca$^{2+}$ overload and ultimately cell death [2,101,154,167].

In conclusion, the measurements of matrix volume and $\Delta \Psi_m$ have considerably advanced our understanding of the possible functions of mitochondrial K+ channels. It is likely that in fully energized mitochondria opening of K+ channels has relatively little effect, whereas under metabolic stress relatively small decreases in $\Delta \Psi_m$ can translate into large decreases of matrix Ca$^{2+}$.

### 3.6. Probing for mitochondrial $K_{ATP}$ channel subunits

A number of different laboratories have pursued the question of whether the mKATP channel may be composed of one of the pore-forming α-subunits of sKATP channels (Kir6.1 or Kir6.2) in combination with an unidentified β-subunit. Using dominant-negative constructs of either Kir6.1 or Kir6.2, Seharaseyon et al. [169] observed that in rabbit cardiac myocytes with reduced functional expression of Kir6.1 or Kir6.2 diazoxide increased flavoprotein fluorescence (used as an assay for functional mKATP channels) to the same extent as in control cells. On this basis, the authors concluded that neither Kir6.1 nor Kir6.2 are components of the mKATP channel. Studies using either the Kir6.1- or Kir6.2-knockout mouse led to the same conclusion since diazoxide increased flavoprotein fluorescence to a similar extent in wild-type and knockout mice [170,171].

In accord with the above studies, confocal fluorescence imaging studies have shown that GFP-tagged Kir6.2, expressed in HEK cells or myocytes, is not targeted to mitochondria [172]. Using a similar method, Hambrock et al. [173] showed that GFP-tagged Kir6.1, alone or co-expressed with various splice forms of SUR1, is not targeted to the mitochondria of COS cells. Likewise, Seharaseyon et al. [169] showed that an anti-Kir6.1 primary antibody labeled the sarcolemma of myocytes but not the mitochondria. In contrast, an earlier electron microscopy study had reported that immunogold labeled Kir6.1 was present in mitochondria [174]. However, Grover and Garlid [100] stated that the antibody used in this study does not react with reconstituted and functional mKATP channels, whereas it does bind to unidentified proteins, suggesting that the results of Suzuki et al. [174] may have been “false-positive.”
Using antibodies and confocal microscopy, Singh et al. [175] reported that Kir6.1, as well as Kir6.2, subunits were localized to mitochondria of rat ventricular myocytes. In a complementary study using immunoblotting and immunogold electron microscopy, Lacza et al. [176] found that both anti-Kir6.1 and anti-Kir6.2 labeled isolated mitochondria. However, mitochondrial proteins were enriched only by a factor of 8 in the Western blots, thus contamination with surface membrane proteins is likely. These authors also reported that Kir6.1 and Kir6.2 harbored mitochondrial targeting sequences. However, we could not verify this. With the most recent versions of MITOPROT (http://mips.gsf.de/cgi-bin/proj/medgen/mitofilter), PREDOTAR (http://genoplante.info.infobiogen.fr/predotar/predotar.html) and TargetP only low prediction scores for mitochondrial localization were obtained. One of the problems with immunocytochemical studies is that successful preabsorption experiments do not rule out cross-reactivity of the antibodies. To ascertain possible mitochondrial localization of the pore-forming Kir6.1 and Kir6.2 subunits, control experiments with Kir6.x knockout mice are mandatory.

In the case of the regulatory SKATP channel subunit SUR, Singh et al. [175] found that anti-SUR2A labeled mitochondria in myocytes. Similarly, Lacza et al. [176] reported that anti-SUR2, but not anti-SUR1, labeled the mitochondrial inner membrane. However, the anti-SUR2 labeled protein had a much smaller molecular mass than SUR2 (~25 versus ~140 kDa; [177]) and co-immunoprecipitation experiments did not reveal any protein-protein interaction between the SUR candidate and Kir6.x subunits. Thus, the 25 kDa SUR-like component described may not be associated with any K⁺ channel.

In conclusion, immunohistochemical and other localization studies have not contributed much to the molecular identification of mKATP channels. There is no convincing evidence that Kir6.x or SURx subunits are localized to mitochondria.

4. The pharmacology of mitochondrial KATP channels

4.1. Openers of mKATP channels

One of the most noteworthy features of mKATP channel pharmacology is that virtually every drug that opens these channels also activates SKATP channels in cardiomyocytes and/or in vascular smooth muscle. To date, the list of drugs which activate both mitochondrial and sarcolemmal KATP channels include: pinacidil, cromakalim, P1060, P1075, minoxidil, KRNN391, sidenafil, desflurane, isoflurane, sevoflurane, aprikalim and levosimendan [4, 178–182]. Notable exceptions are diazoxide and nicorandil, which are thought to activate selectively mKATP channels [183] in cardiac myocytes (but also activate SKATP in other cell types). Sato et al. [183] reported that Nicorandil (100 µM) increased flavoprotein fluorescence, used as an index of mKATP channel activity, without acting on SKATP channels in myocytes, except at high concentrations. In contrast, Tsuchida et al. [184] found that nicorandil-induced cardioprotection was attenuated by the selective SKATP blocker HMR1089, whereas Critz et al. [185] found that pinacidil but not nicorandil protected isolated myocytes from simulated ischemia. Hence, nicorandil may not be such a selective drug as it first appeared. Indeed, [14C]-nicorandil was found to be de-nitratated by cardiac mitochondria [186], yielding the metabolite SG-86 and NO, which complicates the interpretation of studies using this drug.

Another drug, the benzopyran derivative BMS-191095 has been reported to have a cardioprotective effect with relatively little vasorelaxant potency and no effect on the cardiac action potential [187, 188]. Grover and Atwal [187] suggested that BMS-191095 induced the opening of mKATP channels, based on the observation that the cardioprotective effect of the drug was not seen in the presence of glibenclamide or 5-HD. Thus, BMS-191095 may be a good substitute for diazoxide but more studies are required to establish its potential usefulness.

The most commonly used drug for pharmacological preconditioning, diazoxide, has been reported to activate mKATP channels in intact rat liver mitochondria with a Kₐ value of 2.3 µM under particular conditions [97]. These conditions include the presence of 0.5 mM ATP and 1 mM Mg²⁺. Garlid et al. [97] confirmed these data using partially purified mitochondrial proteins reconstituted in liposomes, which contained a fluorescent indicator to estimate K⁺ flux; diazoxide activated K⁺ influx with a Kₐ value of about 0.4 µM in channels obtained from either liver (in the presence of 0.5 mM ATP) or heart (in the presence of 2 mM ATP). One interpretation of the high binding affinity of diazoxide and low sensitivity to millimolar ATP is that the putative mKATP channel is not composed of the known SURs and Kir6.x building blocks. In support of this notion the mKATP channel is, surprisingly, inhibited by ADP and long-chain acyl-CoA esters [189], but not by TEA [96].

The low Kₐ value for diazoxide has been used as evidence to implicate mKATP channels in the mechanism of preconditioning. The mechanism of cardioprotection by diazoxide has, in a way, simply been attributed to the site of action with the highest binding affinity. The highly lipophilic drug diazoxide, though, has been routinely used in preconditioning studies with myocytes and whole hearts at concentrations of 50–100 µM. Thus, other targets of diazoxide, of which there are many, may also come into play during such studies.

In the whole heart, for example, diazoxide potently activates endothelial and smooth muscle KATP channel isoforms. It also weakly activates the cardiac muscle type of SKATP channels, an effect which would be greatly augmented by ischemia due to the concomitant increase in the ADP/ATP ratio [190]. Aside from these KATP channel-related actions, diazoxide is known to inhibit succinate dehydrogenase [147, 155, 160, 161, 191], to act as a protonophoric uncoupler [164], to promote the inhibition of ATP-synthase [192] and to inhibit other nucleotide-requiring enzymes [161].
Another pharmacological approach used to characterize the role of mK<sub>ATP</sub> channels in preconditioning was to compare the relative potency of different K<sup>+</sup> channel openers in cardioprotection and in activation of sK<sub>ATP</sub> channels [94]. For example, Garlid et al. [94] reported that application of diazoxide or cromakalim improved post-ischemic functional recovery in isolated perfused rat and rabbit hearts subjected to global ischemia with approximately equal potency. Since they found diazoxide to be significantly less potent than cromakalim in increasing sarcolemmal K<sup>+</sup> currents they concluded that the effects were due to opening of mK<sub>ATP</sub> channels. In contrast, Haruna et al. [41], analyzing IPC in rabbits by measuring infarct size, found that cromakalim was much more potent than diazoxide in limiting infarct size and contractile dysfunction. In these experiments light scattering was used as an indicator of mitochondrial matrix swelling, and matrix swelling was used as a measure of K<sup>+</sup> influx. As mentioned earlier (Section 3.5), the authors proposed that the main effect of K<sup>+</sup> channel openers in intact mitochondria, with K<sup>+</sup>/H<sup>+</sup> exchange and P<sub>i</sub>/OH<sub>i</sub> exchange in place, was an increase in matrix volume.

In contrast, Das et al. [148], who also used rat heart mitochondria and employed very similar experimental conditions, reported that neither diazoxide (50 µM) nor 5-HD (50 µM) had any effect on light scattering or matrix volume measured using mitochondrial entrapment of radioactive markers. They were “unable to offer an explanation for why [their] data differ from those of Garlid and colleagues” [148]. Interestingly, 5-HD alone, applied at higher concentrations (100 or 300 µM), was found to elicit an increase in matrix volume before and during ischemia, and a decrease in respiration rate during reperfusion [147]. The increase in matrix volume is contrary to what would be expected for a mK<sub>ATP</sub> channel blocker, and the decrease in respiration rate may be related to metabolic effects of 5-HD [156,157]. Taken together, the findings summarized here strongly suggest that 5-HD has effects on mitochondrial energy metabolism independent of its action on mK<sub>ATP</sub> channels [147,148,155–157].

In support of the hypothesis that 5-HD is a specific blocker of mK<sub>ATP</sub> channels, Liu et al. [95] reported that 5-HD reversed the increase in flavoprotein fluorescence induced by diazoxide in rabbit myocytes deprived of metabolic substrate. However, these supportive data rely on the assumption that flavoprotein fluorescence is an unequivocal indicator of mK<sub>ATP</sub> channel activity, which clearly is not the case [155]. Using analytical HPLC and mass spectrometry, Hanley et al. [155] showed that 5-HD, like physiological fatty acids, is activated via acyl-CoA synthetase. Using intact heart and liver mitochondria, Lim et al. [147] confirmed that 5-HD is activated to 5-HD-CoA. Moreover, these authors showed that liver, but not heart, mitochondria can metabolize 5-HD, whereas neither heart nor liver mitochondria appeared to be able to metabolize 5-HD-CoA. Lim et al. [147] suggested that extra-mitochondrially activated 5-HD (5-HD-CoA) cannot enter the matrix via carnitine palmitoyltransferases (CPTI/CPTII). In further work, Hanley et al. [156] demonstrated that 5-HD-CoA, once in the matrix, serves as substrate for at least the first two enzymes of the β-oxidation pathway. Up to this point, it appeared that heart mitochondria cannot take up 5-HD-CoA synthesized on the outer membrane. However, we have recently observed that purified 5-HD-CoA is indeed a substrate for intact heart mitochondria, albeit it is metabolized much more slowly than decanoyl-CoA, probably due to slow kinetics at the penultimate step of β-oxidation [157]. The slow rate of metabolism, and confounding reactions such as acyl-CoA oxidase activity, may have masked the metabolism of 5-HD-CoA in the study of Lim et al. [147].

The notion that 5-HD selectively blocks mK<sub>ATP</sub> but not sK<sub>ATP</sub> channels (Kir6.2/SUR2A) in cardiomyocytes is disputable. Although some groups have reported that 5-HD does not inhibit sK<sub>ATP</sub> channels activated by K<sup>+</sup> channel openers such as cromakalim and pinacidil [98,193], others have shown that 5-HD blocks various surface K<sub>ATP</sub> channels, including Kir6.1/SUR1 and Kir6.2/SUR1, indicating that it has poor mitochondrial specificity [194]. Moreover, Notsu et al. [195] found that 5-HD blocked sK<sub>ATP</sub> channels in cardiomyocytes subjected to metabolic inhibition. This suggests that 5-HD may be able to block sK<sub>ATP</sub> channels under ischemic conditions. Examination of the effects of 5-HD on single-channel activity of sK<sub>ATP</sub> channels may help to clarify this issue.

Glibenclamide has been widely assumed to block both sK<sub>ATP</sub> channels and mK<sub>ATP</sub> channels. However, this drug has K<sub>ATP</sub> channel-independent actions as well. Glibenclamide has been shown to reduce the rate of fatty acid oxidation by inhibiting carnitine palmitoyltransferase [196–199]. At higher concentrations, it has also been shown to block ABC transporters [200,201] and chloride channels [202,203]. Whether such inhibitory actions play a role in the ability of glibenclamide to block preconditioning remains to be tested. In addition, glibenclamide has been reported to uncouple rat liver mitochondria [204], which may explain the increase in flavoprotein fluorescence reported by Liu et al. [95] (see Section 3.4).
The uncoupling induced by glibenclamide may, in fact, not be due to enhanced proton permeation but, instead, to permeabilization of the mitochondrial inner membrane to $\text{Cl}^-$, which promotes net $\text{Cl}^-/\text{K}^+$ influx and dissipates the mitochondrial membrane potential [205].

On balance, the findings summarized in this section strongly suggest that neither diazoxide nor 5-HD are ‘selective’ drugs in the sense that they only activate or block $\text{mK}_{\text{ATP}}$ channels. Since both substances have $\text{K}_{\text{ATP}}$ channel-independent actions in the range of concentrations commonly used for preconditioning they can no longer be considered as arbitrators of the involvement of $\text{mK}_{\text{ATP}}$ channels in cardioprotection. Therefore, alternative mechanisms should be taken into consideration when interpreting the effects of diazoxide and 5-HD on cell survival or functional recovery after ischemia, hypoxia or metabolic stress (see Section 8).

5. Do mitochondrial K$^+$ channels play a role in preconditioning?

5.1. Three candidate K$^+$ channels in the mitochondrial inner membrane

Are $\text{mK}_{\text{ATP}}$ channels involved in preconditioning? Due to the lack of specificity of the most important openers, diazoxide and pinacidil, and the most important blockers, 5-HD and glibenclamide, we must admit that we just do not know. The two K$^+$ channel openers can induce uncoupling in the relevant concentration range [164], and uncoupling per se, for example by DNP, has been shown to confer cardioprotection [164]. 5-HD has been shown to be activated to 5-HD-CoA and to be metabolized by $\beta$-oxidation, with a bottleneck at the penultimate step [157]. Thus, it can interfere with the $\beta$-oxidation of endogenous fatty acids or be used, under certain conditions, as a weak metabolic substrate. Therefore, the notion that a process that is activated by diazoxide and inhibited by 5-HD is necessarily related to $\text{mK}_{\text{ATP}}$ channels is no longer tenable.

The case is made more difficult by the fact that we do not know the gene(s) that may code for the channel. It is unlikely that Kir6.x or SURx channels are involved, and the new concept of the $\text{mK}_{\text{ATP}}$ channel as a protein complex consisting of at least five proteins including ANT and SDH [137] is associated with many imponderables. One problem is the lack of K$^+$ selectivity. If the channel is not K$^+$-selective and is only moderately influenced by ATP, and by the presumed $\text{mK}_{\text{ATP}}$ channel openers and blockers, why should we call it a $\text{K}_{\text{ATP}}$ channel? Another problem is the possible relation to the mitochondrial permeability transition pore, of which ANT may be an essential component (Section 8.3). Furthermore, the measurements of single-channel activity in mitoplasts ascribed to $\text{mK}_{\text{ATP}}$ channels are not at all convincing (Section 3.1). Thus, as long as we do not know the gene and the characteristics of the single channels we cannot be certain that $\text{mK}_{\text{ATP}}$ channels exist.

On the other hand, the case of the $\text{mK}_{\text{ATP}}$ channel is, paradoxically, strengthened by the discovery of BK$_{\text{Ca}}$ channels in cardiac mitochondria [130,132,143]. The similarity of the effects of NS1619 and diazoxide on flavoprotein fluorescence [123] lends some support to the mechanistic concept that flavoprotein fluorescence, under certain conditions, can be used as an indicator of the rate of electron transfer by the respiratory chain (Section 3.4). Although we do not know the targeting sequence directing the channel to the mitochondria, the evidence for the expression of BK$_{\text{Ca}}$ channels in the mitochondrial inner membrane is stronger than the evidence for expression of $\text{mK}_{\text{ATP}}$ channels. Firstly, because there is a candidate gene, and, secondly, because two groups have obtained plausible single-channel measurements [130,132]. Pharmacological preconditioning of the isolated perfused rat heart with NS1619, an opener of BK$_{\text{Ca}}$ channels, produced the same extent of cardioprotection as PPC with diazoxide in the same model. Furthermore, ischemic preconditioning was blunted [123,206] or abolished [207] by blockers of BK$_{\text{Ca}}$ channels. The blockers were effective only when they were present during the trigger phase of preconditioning, they were ineffective when applied only during the index ischemia [206,207]. These findings support the idea that an increase in the K$^+$ conductance of the mitochondrial inner membrane is an essential element of ischemic preconditioning.

Another complication, relevant for the interpretation of previous data, was introduced by the recent identification of Kv1.3 as a mitochondrial K$^+$ channel by Gulbins and co-workers [129]. Using patch-clamp measurements in mitoplasts and an array of molecular genetic techniques, it was clearly shown that the same channel is expressed both at the cell surface and in the mitochondrial inner membrane [129]. The channel can be inhibited by TEA, margatoxin (MgTx), and the sea anemone (Stichodactyla helianthus) toxin, ShK. Application of MgTx and ShK increased $\Delta\psi_m$ in isolated mitochondria [129]. Furthermore, mitochondria of cells lacking Kv1.3 showed no decrease in $\Delta\psi_m$ and no release of cytochrome $c$ after application of the cytostatic drug actinomycin D [208], in contrast to wild-type cells. Thus, we now have three K$^+$ channels to consider that might be involved in preconditioning (Fig. 3).

5.2. The possible functional role of mitochondrial K$^+$ channels

The classical K$^+$ flux studies in isolated mitochondria [2,101,143,209], together with recent studies using the K$^+$ channel modulators diazoxide, NS1619 and MgTx [4,123,154,162,210], give us some clues about the possible function of K$^+$ channels in the mitochondrial inner membrane. Under physiological conditions, the K$^+$ conductance of the mitochondrial inner membrane is probably small, but not negligible, and is tightly regulated. In fully energized mitochondria, K$^+$ influx is compensated by $\text{H}^+$ extrusion, so that moderate activation of K$^+$ channels probably has little effect on $\Delta\psi_m$ [145,162] and matrix Ca$^{2+}$. In contrast, under meta-
bolic stress activation of a K⁺ conductance may lead to a sub-
stantial depolarization. This could occur (i) when the energy
supply is compromised [154], (ii) when the rate of electron
transfer by the respiratory chain is inhibited, or (iii) when the
mitochondrial matrix is loaded with Ca²⁺ [123,162]. Since
matrix Ca²⁺ is very sensitive to \( \Delta \psi_{\text{int}} \), depolarization will lead
to a reduction in matrix Ca²⁺.

In Fig. 4 we have summarized our current view of the role
of K⁺ channels in a qualitative scheme of the events occur-
ring in cardiac mitochondria during preconditioning. The
model is based on the recent findings obtained with BKCa
channels expressed in the mitochondrial inner membrane,
denoted as mKCa channels here, and has been (speculatively)
extended to include Kv1.3 channels, denoted mKv channels,
as well as mKATP channels. The blue lines represent the time
course of K⁺ influx, \( \Delta \psi_{\text{int}} \), matrix volume, matrix Ca²⁺, and
opening of the MPTP in the absence of preconditioning. The
dotted red lines represent the possible time course of events
under conditions of IPC. Please note that, for simplicity of
presentation, the duration of trigger ischemia and index
ischemia (both shaded in gray), respectively, is not drawn to
scale. The uppermost tracing represents the time course of the
K⁺ conductance provided by opening of mKCa, mKATP
and mKv channels. Their open probability is assumed to be
regulated by PKA and PKC, which are activated during the
transient period(s) of ischemia and the subsequent brief rep-
erfusion phase. PKC [211,212] and PKA [69,70] have long
been known to play a role in preconditioning (see Section
2.2). Sato and co-workers have provided evidence that the
putative mKATP may be regulated by PKC [98] and that the
mKCa channel may be regulated by PKA [123]. Kv1.3 chan-
nels are activated by depolarization, and also via PI3K [213–
215].

We assume that the three K⁺ channels act in concert to
regulate the volume of the matrix, and this may have very
profound effects on mitochondrial function [2,101,216,217].
However, in order to induce swelling, the influx of K⁺ ions
requires a counterion, most likely chloride, although the struc-
ture of mitochondrial Cl⁻ channels is not yet clear [218–
222]. Recently, Juhaszova et al. [122] found that many pre-
conditioning stimuli, including IPC and diazoxide, \( \delta \) opioid
and bradykinin B2 receptors, converge upon a common
mechanism: slight swelling (2.5–4%) of mitochondria. Both
swelling and cardioprotection were abolished by the selective
Cl⁻ channel inhibitor IAA94. Cl⁻ flux dependent swell-
ing of mitochondria caused enhanced substrate oxidation.
Juhaszova et al. [122] speculate that the characteristic
‘memory’ of preconditioning is encoded by the mitochon-
drial swelling, but the upstream mechanisms of mitochon-
drial swelling are still unknown. An obvious way in which
this swelling can be brought about is by activation of K⁺ chan-
nels, followed by K⁺ influx driven by the steep electrical gra-
dient, and subsequent Cl⁻ influx through anion-selective
channels and osmotic influx of water. Therefore it may be that the
upstream mechanisms mentioned above, PKC and PKA, con-
D. Contact sites play a key role in energy coupling with the cyto-
membrane space and leads to disruption of contact sites,
resent an important cardioprotective mechanism.
may prevent excessive matrix contraction, and this may rep-
. Production of ROS under control conditions (blue) and after precondi-
tioning (red) is indicated by circles.
verge on the activation of K⁺ conductances in the mitochon-
drial inner membrane [123].
Once activated by kinases, the three K⁺ channels are regu-
lated by [Ca²⁺]ₘ, Δψₘ and, possibly cytosolic and/or matrix
ATP, respectively (Fig. 4). During the initial phase of ischemia,
Δψₘ is expected to fall. It may transiently rise again during
the brief reperfusion phase, eliciting the release of ROS [211].
Simultaneously, [Ca²⁺]ₘ is expected to rise, thus activating
mKCa. During the index ischemia, Δψₘ decreases, [Ca²⁺]ₘ
rises slowly and ATP falls, thus activating mKv, mKₐCa and
mKₐTP channels. The resulting K⁺ influx will produce a higher
matrix volume during the index ischemia and during the ini-
tial phase of reperfusion [147,223]. In the absence of precondi-
tioning, a strong hyperpolarization of the mitochondrial
inner membrane is expected to occur immediately after reper-
fusion (blue line in Fig. 4), associated with a large Ca²⁺
influx and matrix alkalinization, giving rise to the generation
of ROS [222,225]. After IPC, when the mitochondrial K⁺
channels are activated, the post-ischemic increase in [Ca²⁺]ₘ
will trigger a large K⁺ influx through mKₐCa channels, coun-
teracting the increase in Δψₘ. The compensatory K⁺ influx
may prevent excessive matrix contraction, and this may rep-
resent an important cardioprotective mechanism.
Excessive shrinkage distorts the architecture of the inter-
membrane space and leads to disruption of contact sites
between the inner and outer membranes [2,101,216,217]. The
contact sites play a key role in energy coupling with the cyto-
sol via creatine kinase [226] and in the uptake of fatty acids
[227–229]. We hypothesize that the K⁺ influx activated by precondi-
tioning leads to a higher matrix volume during most
phases of preconditioning. The increase in matrix volume is fol-
lowed by an increase in the rate of oxidative phosphoryla-
tion, especially β-oxidation of fatty acids [122,143]. Open-
ing of mKₐCa channels may be particularly important in pre-
venting the sudden buildup of protonic potential immediately
after reperfusion, as illustrated in Fig. 4, which mitigates the
generation of ROS. The price for this cardioprotection is a
mild uncoupling of the mitochondria.

In conclusion, as a result of the collective effort of the sci-
cientific community to understand preconditioning, some hypo-
ethical mechanisms emerged by which K⁺ channels in the
mitochondrial inner membrane might have a cardioprotec-
tive effect. Preconditioning may activate PKC and PKA, and
this may increase the open probability of mKₐCa, mKv and
mKₐTP channels. Although the complex metabolic changes
occurring during ischemia and reperfusion are incompletely
understood, it appears functionally plausible to have three dif-
ferent K⁺ channels in the mitochondrial inner membrane that
collectively regulate matrix volume during ischemia and rep-
erfusion: (i) a channel that is activated by depolarization
(Kᵥ1.3), (ii) a channel that is activated by a rise in matrix
Ca²⁺ (BKₐCa), and (iii) a channel that is activated by a fall in
cytosolic and/or matrix ATP (the putative mKₐTP channel).
K⁺ release from the matrix via the K⁴/H⁺ antipporter is also
controlled in a very complex way [101]. Thus, regulated
uptake of K⁺ and release from the matrix may allow the fine
tuning of matrix volume, without requiring too much energy
expenditure.

6. Metabolic regulation of surface KₐTP channels
in relation to preconditioning

6.1. History of the role of sKₐTP channels in
preconditioning

In initial studies of preconditioning, sKₐTP channels were
assumed to be the major effectors of preconditioning
[31,106,230]. The shortening of the action potential and the
resulting reduction in Ca²⁺ influx and energy expenditure were
thought to readjust the balance between energy supply and
energy expenditure and thus to protect the heart from ischemic
damage [231]. This notion was questioned when it was shown
that low concentrations of K⁺ channel openers that did not
shorten the action potential were still able to induce cardio-
protection [232–234]. Later experiments using flavoprotein
fluorescence and light scattering of mitochondria led to the
suggestion that mKₐTP channels play a more central role in
 cardioprotection [95,100,104,235]. However, recent findings
brought about some kind of renaissance regarding the role of
sKₐTP channels as mediators of cardioprotection [120,236–
241]. Since sKₐTP channels are now known to be extremely
complex metabolic sensors, it is often difficult to distinguish

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Fig. 4. Schematic diagram of the hypothetical changes of some mitochon-
drial parameters during preconditioning. The trigger ischemia (left) and the
index ischemia (right) are marked by gray shading. Only qualitative changes
are shown, and, for simplicity of presentation, the time axis is not drawn to
scale (the index ischemia is actually much longer than the trigger ischemia).
metabolic effects on sK<sub>ATP</sub> from metabolic effects on mK<sub>ATP</sub>. Thus, a thorough understanding of the factors that control opening of sK<sub>ATP</sub> channels in vivo and an analysis of the pharmacology of sK<sub>ATP</sub> channels under various experimental conditions is a prerequisite for the identification and characterization of the putative mK<sub>ATP</sub> channels. In the following brief summary of the metabolic regulation of sK<sub>ATP</sub> channels we emphasize those aspects that may be relevant for our understanding of preconditioning and thus potentially overlap with the metabolic control of mK<sub>ATP</sub> channels.

6.2. Structure and functional roles of sK<sub>ATP</sub> channels

The octameric structure of sK<sub>ATP</sub> channels, consisting of four pore-forming α-subunits (Kir6.1 or Kir6.2) and four regulatory β-subunits (SUR1 or SUR2), is well known [242–244]. The α-subunits are members of the inward rectifier K<sup>+</sup> channel (Kir) family, whereas the β-subunits are members of the superfamily of ATP-binding cassette (ABC) proteins. The α-subunits determine the permeation properties of the K<sup>+</sup>-selective pore and contain the ATP-binding sites, whereas the interaction between α- and β-subunits regulates the ATP sensitivity of sK<sub>ATP</sub> channels. Heteromeric sK<sub>ATP</sub> channels containing both Kir6.1 and Kir6.2 subunits can form functional K<sub>ATP</sub> channels in expression systems [245], but there are conflicting data concerning the possibility that such heteromultimers exist in vivo [245–247]. However, Yoshida et al. [248] have now shown that Kir6.1, Kir6.2 and SUR2B can form heteromultimeric channels in coronary endothelial cells. The ability of Kir6.x subunits to form heteromeric channels implies that a large number of K<sub>ATP</sub> channel proteins with different functional characteristics may exist, as is the case for Kir2.x channels [249]. The number of possible sK<sub>ATP</sub> channels is further increased by alternative splicing of the β-subunits. SUR2A and SUR2B, the main splice variants of the SUR2 gene, show different pharmacological profiles. Various other splice variants of SUR1 and SUR2 have been found, some of which form functional channels when co-expressed with Kir6.x subunits [173,250,251]. Little is known about the pharmacology of these splice variants.

The primary function of sK<sub>ATP</sub> channels is to link cellular metabolism and membrane excitability [252]. Cardiac sK<sub>ATP</sub> channels probably consist of Kir6.2 and SUR2A [252] and provide for a decrease in energy expenditure of the heart by shortening the cardiac action potential and reducing Ca<sup>2+</sup> influx through L-type Ca<sup>2+</sup> channels [31,104,231,253–255] as well as through the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger [256]. The reduction of Ca<sup>2+</sup> influx is particularly important during the initial phase of reoxygenation and is thought to prevent Ca<sup>2+</sup> overload of the mitochondria [241,256–258]. Opening of sK<sub>ATP</sub> channels also prevents arrhythmias and sudden death during vigorous sympathetic stimulation [259].

Pancreatic sK<sub>ATP</sub> channels consist of Kir6.2 and SUR1 [260] and regulate insulin secretion in pancreatic β-cells as well as the excitability of certain types of neurons in the central nervous system. Complexes of Kir6.1 and SUR2B are thought to form vascular sK<sub>ATP</sub> channels [261–265], which play an important role in the metabolic regulation of blood flow. In smooth muscle cells the activation of sK<sub>ATP</sub> channels leads to hyperpolarization and subsequent vasodilatation. In microvascular endothelial cells, increased sK<sub>ATP</sub> channel activity may promote the synthesis of NO by increasing transmembrane Ca<sup>2+</sup> influx [264].

6.3. Regulation of sK<sub>ATP</sub> channels by ATP, ADP and phosphatidylinositol phosphates

The defining property of sK<sub>ATP</sub> channels is their inhibition by intracellular ATP. However, it is now clear that sK<sub>ATP</sub> channels are regulated by a host of different mechanisms and that the other mechanisms may in fact be of greater functional importance than regulation by ATP. Even after more than 20 years of research on the function of sK<sub>ATP</sub> channels, it is not quite clear which factors control the open probability of sK<sub>ATP</sub> channels in vivo. In excised membrane patches from cardiomyocytes the ATP sensitivity is very high. The ATP concentration required for half-maximal inhibition (IC<sub>50</sub>) is in the range 10–50 µM, whereas intracellular ATP does not fall below millimolar levels except under very extreme conditions. If the IC<sub>50</sub> value observed in inside-out patches of sK<sub>ATP</sub> channels would also apply for intact cardiomyocytes, one would not normally expect these channels to open at all in vivo. This apparent discrepancy was resolved when it was recognized that the ATP sensitivity of sK<sub>ATP</sub> channels can be decreased by ADP, membrane-associated phosphatidylinositol phosphates, acyl-CoA esters and many other factors, which brings the IC<sub>50</sub> of sK<sub>ATP</sub> channels much closer to the physiological and pathophysiological range of intracellular ATP.

The most important nucleotide for modulation of sK<sub>ATP</sub> channels in vivo is probably ADP [266]. SUR1 and SUR2 possess two nucleotide-binding domains (NBDs), which cooperatively regulate the kinetics of sK<sub>ATP</sub> channel opening [267–269]. Binding of MgADP or MgGDP at NBD2, which is facilitated by binding of ATP at NBD1, promotes opening of sK<sub>ATP</sub> channels in the presence of otherwise inhibitory concentrations of ATP [270]. Hydrolysis of ATP at NBD2 additionally regulates the gating of sK<sub>ATP</sub> channels by inducing conformational changes [269]. Thus, the kinetics of ATP hydrolysis by SUR subunits and the local cytosolic ADP concentration are important factors in determining the function of sK<sub>ATP</sub> channels [271].

Phosphatidylinositol phosphates are not only components of the cell membrane but also important second messengers [272]. PIP<sub>2</sub> activates sK<sub>ATP</sub> channels, probably by binding to a domain at the C-terminus that is in close proximity to or overlaps with the ATP-binding domain [273–277]. The competition between the two ligands results in a shift of the IC<sub>50</sub> for ATP binding, adding to the shift induced by intracellular ADP. Since the concentration of PIP<sub>2</sub> at the inner leaflet of the cell membrane is modulated by G protein-coupled receptors, for example, via activation of PLC or PI3K, the regulation of sK<sub>ATP</sub> channel activity may play an important role in
signal transduction in cardiac muscle [278]. This may be particularly relevant during ischemia, which leads to activation of PLC by various agonists.

6.4. Regulation of $sK_{ATP}$ channels by metabolic intermediates

The local submembrane concentrations of ATP may differ substantially from the ‘bulk concentration’ of ATP in the cytosol [279–281], despite the fact that under physiological conditions the submembrane ATP and ADP concentrations are buffered by the phosphotransfer enzymes creatine kinase and adenylate kinase [18,282]. Inhibition of the sarcolemmal Na⁺,K⁺-ATPase can lead to closure of $sK_{ATP}$ channels by increasing the submembrane concentration of ATP [281,283]. Conversely, the strong activation of the Na⁺,K⁺-ATPase during reperfusion can lead to the opening of $sK_{ATP}$ channels by depleting submembrane ATP [41].

Recent studies indicate that, in cardiomyocytes and in other cell types, $sK_{ATP}$ channels, like other cardiac ion channels [284], may in fact exist as a protein complex [285–287]. The $sK_{ATP}$ channel complex includes creatine kinase, adenylate kinase and the muscle form of lactate dehydrogenase (M-LDH). It was found that the physical association of the $sK_{ATP}$ channel with M-LDH modulates ATP sensitivity [286]. The $sK_{ATP}$ channel is also regulated by the catalytic activity of glyceraldehyde-3-phosphate dehydrogenase; 1,3-bisphosphoglycerate, an intermediate product of glycolysis, directly regulates the $sK_{ATP}$ channel, even in the presence of high cytosolic ATP [288].

Long-chain fatty acids are the most important metabolic substrate of the heart under physiological conditions. The activated form of these fatty acids, long-chain acyl-CoA esters, have been shown to decrease the ATP sensitivity of $sK_{ATP}$ channels in cardiomyocytes with a similar potency as PIP₂ [289], and it has been suggested that these soluble cytosolic molecules interact with $sK_{ATP}$ channels by the same mechanism as PIP₂ [277]. Furthermore, medium- and short-chain acyl-CoA esters have also been shown to reduce the ATP sensitivity of $sK_{ATP}$ channels [290].

Intracellular acidification also decreases the sensitivity of $sK_{ATP}$ channels to ATP [291–295]. The stimulatory effect of intracellular acidification on the open probability of $sK_{ATP}$ channels is particularly large at low cytosolic ATP concentrations, which facilitates channel opening during metabolic inhibition [295]. The ATP sensitivity of $sK_{ATP}$ channels can also be decreased directly by intracellular lactate [296]. Thus, PIP₂, acyl-CoA esters, intracellular protons and lactate, all of which are increased during ischemia, relieve the inhibition of $K_{ATP}$ channels and thus may have a cardioprotective effect.

6.5. Blockers and openers and of $sK_{ATP}$ channels

$K_{ATP}$ Channel blockers selective for $sK_{ATP}$ channels, such as the sulfonilthiourea HMR1098 [297], have been used as tools to explore the role these channel in preconditioning. In several studies it was found that application of HMR1098 at concentrations that can inhibit $sK_{ATP}$ channels did not significantly attenuate IPC or PPC [38,298,299]. However, some forms of IPC and PPC were sensitive to HMR1098 [171,180,184,300,301].

The $K^+$ channel opener P1075, a pinacidil analogue, binds with high affinity to SUR2A and to SUR2B, but not to SUR1, in the presence of MgATP [302–305]. This drug has been proposed to be a selective opener of $sK_{ATP}$ channels and has been used as a tool to clarify the relative contributions of $sK_{ATP}$ and m$K_{ATP}$ channels to preconditioning [121,235,238,241,256]. Baczko et al. [121] showed in a cellular model of cardioprotection that addition of P1075 to the reoxygenation solution reduced the contractile dysfunction and Ca²⁺ overload in rat ventricular myocytes after metabolic inhibition. This effect was blocked by HMR1098 and did not occur in myocytes expressing a dominant-negative Kir6.2 construct, indicating that it was indeed related to opening of $sK_{ATP}$ channels. Similar cardioprotective effects were observed in quiescent cardiomyocytes [241,256]. The authors propose that improved Ca²⁺ homeostasis, due to reduction of reverse Na⁺/Ca²⁺ exchange, is responsible for the beneficial effect of $sK_{ATP}$ channel activation. It should be noted, however, that some researchers questioned the selectivity of P1075 and proposed that the drug also opens m$K_{ATP}$ channels [181,306–308].

Diazoxide is generally considered not to activate cardiac $sK_{ATP}$ channels under normal conditions [232–234]. However, recent experiments on isolated cardiomyocytes exposed to metabolic inhibition have shown that pretreatment with diazoxide led to earlier opening of $sK_{ATP}$ channels, earlier failure of action potentials and earlier contractile failure, which suggests that diazoxide can indeed enhance the open probability of $sK_{ATP}$ channels during metabolic inhibition [239]. The opening of $sK_{ATP}$ channels induced by diazoxide is not necessarily due to a direct effect on the channel but may be partially attributable to uncoupling of mitochondria [164] or inhibition of succinate dehydrogenase [147,155,159,161]. Furthermore, D’Hahan et al. [190] found that the sensitivity of cardiac $sK_{ATP}$ channels to diazoxide increased in the presence of MgADP and during simulated ischemia. Thus, especially during ischemia, where m$K_{ATP}$ channel are assumed to be most important, diazoxide may not be selective enough to serve as a useful tool for identifying m$K_{ATP}$ channels.

7. Do surface KATP channels play a role in preconditioning?

7.1. Changes in action potential duration during metabolic inhibition

Opening of $sK_{ATP}$ channels is expected to be cardioprotective by shortening the action potential and reducing Ca²⁺ influx during ischemia and reperfusion (see Sections 6.1 and 6.2). Gross and Auchampach [31] showed that the protection...
in dog heart induced by IPC could be blocked by glibenclamide and mimicked by the K+ channel opener aprakalin. Similarly, experiments in guinea-pig ventricular muscle showed that application of glibenclamide inhibited shortening of the action potential during ischemia and impaired recovery of ventricular function, whereas the opposite effects were obtained with the K+ channel opener pinacidil [309]. In swine heart, IPC also induced shortening of the action potential during the index ischemia and this was associated with cardioprotection [310]. Both effects were not observed after application of glibenclamide.

Using cell-attached patch-clamp recording in guinea-pig cardiomyocytes, Zhu et al. [114] showed that anoxic preconditioning shortened the time interval between metabolic inhibition and opening of sKATP channels. The effect was associated with decreased ATP sensitivity of sKATP channels and could be abrogated by blocking PKC. Thus, phosphorylation of the channel protein by PKC during hypoxia may modulate its open probability during subsequent metabolic inhibition. As mentioned in Section 6.5, Rodrigo et al. [239] found that diazoxide improved functional recovery of rat cardiomyocytes subjected to metabolic inhibition. The improved recovery was associated with earlier activation of sKATP channels during metabolic inhibition and improved recovery of contractile function after removal of metabolic inhibition. Extending this work, Rainbow et al. [240] have used isolated rat cardiomyocytes transfected with a C-terminal fragment of SUR2A (residues 1294–1358). After 2 days in culture, the SUR2A fragment had reduced sarcolemmal KATP currents to 15% of control. In transfected cells, action potential failure during metabolic inhibition was delayed, Ca2+ loading of the cells was increased and the cardioprotective effect of PPC with DNP was abolished. These findings are consistent with a major role of sKATP channels in PPC in the rat.

7.2. Evidence from experiments with transgenic mice

The possible involvement of sKATP channels in cardioprotection was also tested with Kir6.2 knockout mice. It was found that knockout of Kir6.2 abolished the protective effects of IPC [171,311,312]. The recovery of contractile function of isolated perfused hearts from Kir6.2 knockout mice was significantly impaired in comparison to control animals. Interestingly, the cardioprotective effect of pharmacological preconditioning with diazoxide was also absent in Kir6.2 knockout mice [300]. In wild-type mice, PPC with diazoxide and IPC could be prevented by blocking sKATP channels with the surface-selective KATP channel blocker HMR1098, but the putative mKATP channel blocker 5-HD was ineffective [171,300].

Gumina et al. [312] studied the adaptation of energy metabolism to IPC in wild-type and in Kir6.2 knockout mice. They found that in wild-type mice the rates of ATP synthesis and ATP hydrolysis were better preserved after preconditioning, resulting in improved ionic homeostasis and contractility, confirming earlier reports [104,313,314]. The beneficial effects of preconditioning on energy metabolism were absent in the Kir6.2 knockout mice [312]. These findings suggest that the sensing of metabolic deficits and the adaptation of electrical and mechanical activity to the overall metabolic situation, which is normally provided by sKATP channels, is an essential component of preconditioning. Taken together, the experiments with Kir6.2 knockout mice provide strong evidence for involvement of sKATP channels in IPC and PPC, at least in the mouse [315–317]. Since the transfer of energetic signals between different intracellular compartments, to which sKATP channels contribute [18], is probably similar in all mammals, it is likely that the sKATP channels contribute to stress tolerance also in the heart of larger mammals, including humans [271]. It should be mentioned, however, that some studies came to the conclusion that the sKATP channels do not contribute to preconditioning in larger mammals. This view was mainly based on the observation that the sKATP-selective K+ channel blocker HMR1098 was ineffective at inhibiting IPC or PPC with larger mammals [38,166,298,299]. However, in these studies the inhibitor was not present during index ischemia and reperfusion, when sKATP channel activity most likely effects cardioprotection.

Kir6.1 channels are also expressed in cardiac muscle [264,318]. However, the sKATP channel activated by pinacidil in mouse ventricular myocytes does not seem to be affected by deletion of the gene coding for Kir6.1 channels [170]. Thus, Kir6.1 channels are unlikely to be a constituent of sKATP channels. In addition, the channel is unlikely to be involved in the formation of mKATP channels, since in Kir6.1 knockout mice a similar increase in flavoprotein fluorescence was elicited by diazoxide and inhibited by 5-HD as in wild-type mice [170]. In contrast, knockout of Kir6.1 abolished glibenclamide-sensitive KATP channel current in vascular smooth muscle. In conclusion, the physiological role of Kir6.1 in cardiac muscle remains unclear [319], but the Kir6.2 knockout experiments provide strong support for a role of surface KATP channels in preconditioning.

7.3. Effects of adenosine on sKATP channels

Adenosine is a special mediator of preconditioning because, unlike acetylcholine, bradykinin, opioids and phenylephrine, it affords preconditioning without stimulating the release of ROS [11,40] and without causing mitochondrial swelling [122]. Extracellular adenosine accumulates in the heart during ischemia, inducing cardioprotection [320–322]. One of the most important mediators of adenosinergic cardioprotection is PKC [52]. Activation of PKC in cardiomyocytes increases the open probability of sKATP channels, most likely by phosphorylating Kir6.2 at threonine 180 [257,322–327]. Thus, opening of sKATP channels may contribute both to PPC and to IPC [257,322,328].

It has now become clear that the surface expression of receptors, channels and transporters is subject to regulation of trafficking, although in many cases the signal transduction pathways modulating biosynthetic forward trafficking or
endocytosis are still unclear [329]. In a pioneering study, Hu et al. [330] have shown that incubation of cardiomyocytes with adenosine for 30 min downregulates the surface expression of sKATP channels in a PKC-dependent manner. This effect was due to enhanced endocytosis mediated by PKC, and it may serve to limit the activating effect of adenosine on sKATP currents [330]. Modulation of the number of functional KATP channels at the surface of cardiomyocytes via PKC or via other mechanisms [120] may also play a role in preconditioning, although it would antagonize the early effects of PKC on the open probability of sKATP channels and would increase action potential duration.

7.4. Effects of preconditioning on surface expression of sKATP channels

Immunoprecipitation, Western blot and immunofluorescence experiments with guinea-pig cardiomyocytes showed that preconditioning with a 5 min episode of hypoxia induced recruitment of additional sKATP channels to the sarcolemma [120]. At the beginning of the sustained (index) hypoxia the number of functional sKATP channels at the cell surface was increased by a factor of ~6 as compared to control cardiomyocytes. The authors suggested that the cardioprotective effect observed after the 5 min episode of hypoxia was due to opening of sKATP channels at the beginning of the sustained hypoxia. Cardioprotection was abrogated in the presence of glibenclamide or 5-HD, as well as in the presence of the selective sKATP channel blocker HMR1098. It may be concluded that, at least in the model of reperfusion damage after a brief conditioning period of hypoxia, sKATP channels may play a major role in extending the period that cardiomyocytes can survive during hypoxia in the guinea pig.

7.5. The contribution of other channels to K+ efflux during ischemia

The massive K+ efflux from cardiomyocytes observed during ischemia or hypoxia [331,332] cannot be explained on the basis of KATP channel opening alone but requires an equal flow of a counter-ion [333–335]. One possibility is that K+ efflux is at least partially balanced by Na+ influx [334,336]. Another possibility is that the K+ efflux observed during myocardial ischemia is accompanied by a substantial efflux of anions through volume-activated Cl− channels [337]. An interesting hypothesis concerning the role of K+ channels in preconditioning has recently been put forward by Diaz et al. [338]. They suggested that the beneficial effect of K+ channel opening may be related to the fact that regulatory volume decrease mediated by Cl− efflux requires concomitant K+ efflux to be effective. They found that blockade of Cl− channels impaired both IPC and PPC [339,340], and they postulated that this was due to inhibition of regulatory volume decrease. Furthermore, they found that IPC diminished cell swelling induced by ischemia and proposed that improved cell volume regulation plays a significant role in the protection afforded by IPC. In agreement with this hypothesis, they observed that inhibition of inward rectifier K+ channels in cardiomyocytes abolished cardioprotection induced by IPC [338]. Furthermore, genetic or pharmacological inactivation of Cl− channels also prevented ischemic preconditioning [341]. Thus, it appears possible that one of the functions of sKATP channel opening is to facilitate volume-activated Cl− efflux.

In conclusion, there is no doubt that sKATP channels make a substantial contribution to preconditioning, in agreement with the original ideas put forward by Gross and Auchampach [31]. The most convincing pieces of evidence come from the results obtained with Kir6.2 knockout mice [171,300,312] and from the recent studies on the regulation of surface expression of sKATP channels in guinea-pigs by IPC [120]. The role of sKATP channels can be explained by the fact that these channels are integrative sensors of metabolic intermediates and adapt the electrical activity to the energy status of the cardiomyocytes. This adaptive response is particularly important under conditions of stress, such as hypoxia and reperfusion, but it may also play a role in human disease [271]. Enhanced cell volume regulation during index ischemia, mediated by the opening of sKATP channels and volume-activated Cl− channels, may be one of the key downstream mechanisms for cardioprotection through preconditioning [340,341].

8. Alternative mechanisms of preconditioning

It is now clear that several mechanisms cooperate to induce cardioprotection. The most important mechanisms, apart from the opening of K+ channels, are generation of ROS, changes in fatty acid metabolism and opening of the mitochondrial permeability transition pore (MPTP). The different mechanisms are most probably all interdependent. For clarity, we discuss the three alternative mechanisms separately (Sections 8.1–8.3) and then try to present an integrated picture of the mechanisms underlying preconditioning (Section 8.4).

8.1. The role of ROS production in preconditioning

Reactive oxygen species (ROS) have been incorporated in most conceptual schemes of preconditioning. During normal respiration, approximately 2–4% of electron flow through the respiratory chain results in only partial reduction of O2, generating superoxide anions [342,343], most likely at complexes I and III [226,344]. Increased ROS production takes place during ischemia [345–348], causing damage to the electron transport chain [348–350]. During reperfusion there is a burst of ROS production that may inflict massive cardiac damage [224,226,351]. The loss of function of the respiratory chain and the production of ROS progress with increasing duration of the ischemia. As a result, recovery of mitochondrial function during reperfusion, a prerequisite of cell survival, critically depends on the duration of the preceding ischemia.
ROS play both a “good” and a “bad” role in relation to preconditioning [2–4,352,353]. On one hand, preconditioning is thought to reduce the massive ROS production which accompanies reperfusion after index ischemia. Thus, inhibition of ROS production may represent an end effector of IPC and PPC. On the other hand, low concentrations of ROS are well-recognized intracellular messengers [351,354–357], and have been proposed to represent a trigger for IPC, and even PPC, by switching on cardioprotective mechanisms. However, exactly when, where and how ROS increase under physiological and pathophysiological conditions is still a controversial issue. This is mainly due to, first, the inherent limitations of the fluorescent dyes used to monitor ROS in intact cells and, second, the potentially misleading results of experiments with isolated mitochondria, submitochondrial particles or purified enzyme systems [358], in which ROS can be measured more directly.

A high electrochemical proton gradient across the mitochondrial inner membrane has been reported to favor the generation of ROS [359–363]. Based on these observations, it has been hypothesized that ROS production can be decreased by uncoupling and subsequent depolarization of the mitochondrial inner membrane [363,364]. Uncoupling can be provided by uncoupling proteins or long-chain fatty acids [168,365,366], and this may represent a protective mechanism preventing excessive production of ROS. Many of the commonly used mKATP channel openers are highly lipophilic and capable of transferring protons across the mitochondrial inner membrane [145,159,164]. Specifically, diazoxide and pinacidil have been shown to promote an H+-selective current across lipid bilayers, and both agents increased state-4 respiration in isolated heart mitochondria to a similar extent when K+ was substituted with Na+ [164].

Interestingly, the classic uncoupler DNP, similar to diazoxide and pinacidil, also protects hearts from ischemia, assessed by measuring mechanical recovery after 45 min of global ischemia [164,165]. These data suggest that protonophoric uncoupling itself can precondition the heart, and that diazoxide could in principle limit ROS production by its uncoupling action during reperfusion after index ischemia or hypoxia [212,224,367,368]. Consistent with this idea, diazoxide has been shown to prevent fatty acid induced, ROS-mediated loss of cytochrome c from isolated mitochondria [168]. However, the molecular mechanism by which enhanced ROS production might trigger cytochrome c loss is still under debate [40,122,168,350,359,370].

As mentioned above, ROS generated during IPC appear to represent an important upstream mechanism that is required for the cardioprotective effect of the trigger ischemia [56,102,371]. One of the principal downstream effectors of ROS is PKCε [58,62,63,66,109,211,212,372,373], which may be translocated to the mitochondria [68]. Cardioprotection conferred by IPC, as well as PPC, can be prevented by application of ROS scavengers before the index ischemia [37,82,84,347] or by the inhibition of PKCε [34]. Oldenburg et al. [102] and others [44,102,374] have proposed that opening of mKATP channels is the primary mechanism, and that generation of ROS is downstream of mKATP. This hypothesis was based on the observation that diazoxide [181,375], volatile anesthetics [13,44,87] and acetylcholine can increase ROS production. In contrast, using malondialdehyde as an indicator of ROS. Dzeja et al. [161] reported that brief exposure to 30 µM diazoxide reduced rather than increased ROS production, whereas an increase in ROS production occurred after washout of diazoxide. The inferences of Oldenburg et al. [102] were based on the assumption that diazoxide and 5-HD act selectively on mKATP channels, which we no longer share. In view of the difficulty of measuring ROS in intact cells, we think it is also possible that generation of ROS is the primary event during the trigger phase of preconditioning and that cardioprotection results from subsequent activation of PKCε and other downstream enzymes.

When one examines the expanding list of drugs that afford cardioprotection, a common mechanism of action emerges: diazoxide and 3-nitropropionic acid inhibit succinate dehydrogenase (complex I), volatile anesthetics and pinacidil inhibit complex I and, finally, nicorandil can inhibit complex IV. Most of the cardioprotective drugs inhibit the respiratory chain to some extent. This might explain why such structurally diverse drugs share the ability to induce cardioprotection. Although the ability of these drugs to block partially the respiratory chain is not disputed, and may even be dismissed as a (predictable) toxic side effect [2,144], the possibility that pharmacological inhibition of the respiratory chain, like ischemic inhibition, can trigger cardioprotective mechanisms should be further pursued. The mechanism, by which partial inhibition of the respiratory chain may afford preconditioning has not yet been resolved. It has been proposed, that inhibition of the respiratory chain at more distal sites may enhance the basal rate of ROS generation, because decreased flux through the electron transport chain increases the reduction of proximal sites, enhancing electron leak and superoxide generation [161,226,342,376–378]. This mechanism would be consistent with the hypothesis that ROS act as initial triggers of preconditioning [56].

In conclusion, the double-edged role of ROS in regulating cell death and cell survival is potentially important for understanding preconditioning. There is good evidence that application of ROS scavengers during the trigger phase prevents preconditioning and that excessive production of ROS during the reperfusion phase causes cell injury. There are indications that a moderate increase in ROS production during the trigger phase may initiate preconditioning, but the underlying molecular mechanisms are not yet clear. The targets of ROS during the trigger phase (including PKC, but perhaps also other kinases) require further investigation. One of the most plausible downstream targets of ROS is the mitochondrial permeability transition pore (see Sections 8.3 and 8.4).

### 8.2. The role of fatty acid metabolism in preconditioning

In normoxic cardiac muscle, over 70% of cardiac energy expenditure is related to β-oxidation of fatty acids [379], and
uptake and utilization of fatty acids are finely tuned, presumably to prevent intracellular accumulation of fatty acids, as these are toxic at high concentrations [380]. During ischemia, the myocardial content of lipids strongly increases [379,381,382], glycolysis rates are high and the rates of glucose oxidation are low [383–385]. Thus, during initial ischemia, before the oxygen supply becomes too low, fatty acid oxidation dominates even more than during normoxia [379,386]. The shift away from glucose oxidation to fatty acid oxidation is mainly due to a decrease in malonyl-CoA concentration [387,388], mediated by the rapid rise in AMP during ischemia and the resulting inhibition of acetyl-CoA carboxylase [386,389]. The decrease in malonyl-CoA promotes the uptake of acyl-CoA esters into mitochondria via carnitine palmitoyl transferase I (Fig. 5) or carnitine octanoyltransferase. During prolonged ischemia the paradoxical situation arises that acyl-CoA esters continue to be delivered to the mitochondrial matrix in the absence of a sufficient oxygen supply for β-oxidation [389].

During reperfusion, the levels of malonyl-CoA fall even further [390,386]. Moreover, reperfusion is associated with enhanced degradation of membrane phospholipids, probably mediated by phospholipase A2 [379], which leads to further accumulation of fatty acids [391]. The combination of fatty acid accumulation and low malonyl-CoA levels leads to strong enhancement of fatty acid oxidation at the expense of glucose metabolism, and it has been suggested that the excessive use of fatty acids by the heart during and after ischemia contributes to ischemic injury [168,386,392,393].

Lim et al. [147] have shown that ischemia induces swelling of mitochondria, and that matrix volume is increased even further after 30 min of reperfusion. In earlier work, Halestrap had shown that matrix swelling is associated with an enhancement of fatty acid oxidation [143,394,395]. These results were recently confirmed by Juhaszova et al. [122] who found that mitochondrial swelling was associated with enhanced substrate oxidation (palmitate ≥ octanoate > glucose) and production of ROS. Thus, matrix swelling is expected to further accentuate the dominance of fatty acid metabolism during ischemia and reperfusion.

5-Hydroxydecanoate, a substituted fatty acid, has also been shown to be metabolized by β-oxidation [147,155–157]. Since 5-HD has been widely used as an inhibitor of preconditioning it may be useful to pursue the question to what extent the effects of 5-HD may be related to metabolism of the drug (Fig. 5). For example, it may be speculated that the inhibitory effect of 5-HD on PPC may be related to its β-oxidation, which could act to bypass partial inhibition at either complex I or both complex II [155,156]. This mechanism may be relevant in experimental models in which the PPC is performed in the absence of metabolic substrates.

Since 5-HD has been shown to be activated to 5-HD-CoA [147,155] and metabolized by the β-oxidation pathway [157], it may interfere with the increase in fatty acid oxidation observed during reperfusion. Recent experiments suggest that 5-HD is metabolized much less efficiently than decanoate, and that metabolic intermediates of 5-HD can indeed inhibit fatty acid oxidation [157]. Thus, metabolism of 5-HD could undermine the beneficial consequences of ischemic or phar-
macological preconditioning. Interestingly, Juhaszova et al. [122] found that diazoxide caused an increase in substrate oxidation (associated with matrix swelling) and induced cardioprotection, and that application of 5-HD or the partial fatty acid oxidation inhibitor trimetazidine prevented these effects. The inhibitory effect of 5-HD was most pronounced when palmitate or octanoate were used as substrates. Thus, in view of the partial metabolism of 5-HD via β-oxidation [156,396] an obvious possibility is that 5-HD does not act by blocking the putative mKATP channel but that one of its metabolites, most likely L3,D5-dihydroxydecanoyl-CoA, provides a bottleneck for β-oxidation [147,155,157]. There is another metabolic effect that cytosolic 5-HD-CoA might share with endogenous acyl-CoA esters: inhibition of the ANT [379], which would reduce the rate of oxidative phosphorylation. Lim et al. [147] observed that application of 5-HD increased mitochondrial matrix volume and suggested that this might have been mediated by inhibition of the ANT by the CoA ester of 5-HD [147].

Glibenclamide, another presumed inhibitor of mKATP channels, has been shown to reduce the rate of fatty acid oxidation by inhibiting carnitine palmitoyl transferases [197–199]. At a free concentration of 5 μM, glibenclamide inhibited CPTI/CPTII by about 50%. Hence, both glibenclamide and 5-HD may share the common property of inhibiting fatty acid metabolism. Could such an action block preconditioning? This may indeed be the case, since aside from the study of Juhaszova et al. [122] mentioned above, the β-oxidation inhibitor trimetazidine has been shown to abolish both IPC and PPC, indexed by measuring infarct size relative to area at risk in rat hearts [165]. It may be speculated, therefore, that the substantial changes in fatty acid metabolism induced by ischemia, or even by 5-HD and glibenclamide, may play a pivotal role in preconditioning.

In conclusion, the ability of mitochondria to cope with the massive alterations in fatty acid metabolism after ischemia is a crucial factor for survival of the cardiomyocytes; the excessive use of fatty acids is detrimental to both the ischemic and the reperfused heart [397,398]. Mitochondrial matrix volume modulates fatty acid metabolism even more strongly than carbohydrate metabolism [122,143]. The ‘universal’ blocker of IPC, 5-HD, is a substituted fatty acid that may interfere with fatty acid metabolism at various points, and this is also the case for glibenclamide. Therefore it may be worthwhile to study the modulation of fatty acid metabolism by ischemic and pharmacological preconditioning in more detail.

8.3. The role of the mitochondrial permeability transition pore in preconditioning

The mitochondrial permeability transition pore (MPTP) is a protein complex consisting of the ANT, cyclophilin D, the voltage-dependent anion channel (VDAC) and other proteins [140,142,399–403]. It represents a Ca2+-, Mg2+-, voltage-, pH- and redox-gated channel in the mitochondrial inner membrane [404]. In addition, the MPTP is regulated by adenine nucleotides [403], similar to KATP channels. The conditions for opening the MPTP are optimal during reperfusion after prolonged ischemia [403], and various groups have shown that block of the MPTP with cyclosporin-A (CsA) or sanglifehrin-A (SfA) during reperfusion can reduce the number of necrotic cells [223,405–408]. It is now generally accepted that prolonged opening of the MPTP during the first few minutes of reperfusion is a critical determinant of myocardial cell injury (reviewed in refs. [141] and [403]).

A brief report by Hausenloy et al. [40] provides some evidence that, in addition, the MPTP may be transiently opened during the preconditioning phase (before the index ischemia) and that this may play a role in fostering cell survival. These authors showed that blockade of the MPTP during the preconditioning phase with either CsA or SfA abrogated (i) IPC in isolated rat heart, (ii) PPC with diazoxide, (iii) PPC with the uncoupler DNP, and (iv) PPC with the selective adenosine A1-receptor agonist CCPA. These data suggest that all of these variants of preconditioning are in fact related to transient opening of the MPTP. In the presence of a ROS scavenger the protection afforded by all variants of preconditioning was abolished, except for the protection induced by CCPA [166], in agreement with an earlier study by Cohen et al. [11]. In addition, Hausenloy et al. showed that diazoxide caused loss of the fluorescent probe calcein from mitochondria, which they interpreted to indicate transient opening of the MPTP [40,409,410] because calcein can only cross the mitochondrial inner membrane when the pore opens [369,411,412]. Moreover, the diazoxide-induced reduction of calcein fluorescence was abolished in the presence of CsA or 5-HD [40]. The authors postulate that mitochondrial uncoupling or an increase in matrix pH can induce transient opening of the MPTP, which then promotes the release of ROS [40]. The exact order in which these events occur is not yet clear since, in the converse situation, ROS have been shown to facilitate the opening of the MPTP [142,413], possibly resulting in a positive feedback [369]. The findings of Hausenloy et al. [40] extend previous work showing that transient MPTP opening mediates depolarization and Ca2+ efflux from mitochondria [404], and possibly induces mitochondrial ROS release [369].

Phosphoinositide-3-kinase (PI3K) also appears to be involved in cardioprotection [77–80,414–416]; the downstream mediators of PI3K are PKB/Akt and glycogen synthase kinase-3β (GSK-3β) [78]. Recently, GSK-3β has come into focus as a potential mediator of preconditioning [78,416,417]. The non-phosphorylated form of GSK-3β is active in unstimulated cells, where it phosphorylates several targets (in addition to glycogen synthase), usually resulting in inhibition of these targets [418,419]. GSK-3β can be phosphorylated by various kinases, including PKB/Akt, which inhibits its activity (often stimulating downstream targets). Interestingly, GSK-3β is also localized in mitochondria, together with PKB/Akt [420]. A recent study by Juhaszova et al. [122] on the role of GSK-3β in preconditioning provides some insights into the mechanisms underlying preconditioning. Analyzing changes in mitochondrial permeability transition (MPT), Δψm and mitochondrial swelling, they found.
that various preconditioning stimuli converge upon GSK-3β [122]. They propose that phosphorylation of GSK-3β leads to an increase in the threshold for MPT activation by ROS during reperfusion, which may account for cardioprotection (Fig. 5). In essence, the authors proposed that multiple protective pathways, including PKA, PKB/Akt and PKC, converge on GSK-3β and thereby induce inhibition of the MPTP, a key mediator of cell death.

Based on Fourier analysis of line-scan images, Juhaszova et al. [122] report that exposure to a variety of cardioprotective agents, including diazoxide, was associated with mitochondrial matrix swelling and that this swelling could be reversed or prevented by the Cl⁻ channel blocker IAA94. They also found that mitochondrial swelling was associated with enhanced substrate oxidation (measured as oxygen consumption) and production of ROS (measured with a fluorescent indicator) [122]. The increased ROS production was assumed to induce translocation of PKC to mitochondria [58,66,68], followed by inhibition of GSK-3β [122]. GSK-3β was found to be localized in mitochondria, and experiments with transgenic mice as well as gene silencing with siRNA showed that GSK-3β but not GSK-3α was involved in cardioprotection. The most significant finding of this study is the characterization of the shift in the threshold for ROS activation of the MPTP that can be elicited by various forms of preconditioning [122]. The conclusions of Juhaszova et al. are consistent with the observation that IPC and a variety of cardioprotective drugs increase the delay between ischemic insult and reperfusion damage [223,405,421,422] or ROS-induced loss of Δψ [166].

Multiple studies have shown that activation of PKCe and translocation of the enzyme to mitochondria is critical for preconditioning [54,62,372]. Using co-immunoprecipitation from mouse cardiac mitochondria, Baines et al. [68] have now shown that PKCe can interact with and phosphorylate the VDAC, a component of the MPTP. Furthermore, using measurements of mitochondrial swelling and transgenic mice overexpressing PKCe, they obtained indirect evidence that phosphorylation via PKCe inhibits opening of the MPTP. Thus, the MPTP may be a downstream target of a variety of cardioprotective stimuli that inhibit GSK-3β or stimulate PKCe.

In conclusion, the experimental data summarized in this section suggest that the MPTP is a crucial element of the endogenous cardioprotective program. Indeed, the MPTP may be one of the elusive “end effectors” of PPC and IPC. Whether transient opening of the MPTP is, in addition, a “trigger” for ischemic preconditioning remains to be elucidated.

8.4. Bringing it all together

As we have shown in the preceding sections, several mechanisms contribute to the endogenous cardioprotective program: (i) It is likely that K⁺ channels in the mitochondrial inner membrane, perhaps including mKATP, contribute to preconditioning (see Section 5). (ii) There is no doubt that sK_ATP channels contribute to preconditioning (see Section 7). (iii) Since IPC and PPC are prevented by ROS scavengers, the generation of ROS appears to be essential for preconditioning. (iv) The ability of mitochondria to cope with the massive alterations in fatty acid metabolism after ischemia is certainly also a crucial factor for the survival of cardiomyocytes (see Section 8.2). (v) The MPTP probably plays a decisive role in mediating myocardial injury during reperfusion (Section 8.3).

How can these mechanisms be integrated into a coordinated response of the cell to ischemia and reperfusion, or metabolic stress in general? A plausible hypothesis for the mechanism of action of ROS is that they activate PKC [109,211,212], and possibly other kinases, which then leads to an increased open probability of K⁺ channels in the mitochondrial inner membrane lasting for 1 h or more (see Section 5.2). The opening of the K⁺ channels is cardioprotective, because, in essence, it produces a mild uncoupling that mitigates hyperpolarization and ROS release during a subsequent sequence of ischemia and reperfusion. Put in a very simple way, the generation of ROS in cardiomyocytes activates an adaptive response that diminishes ROS generation in the next few hours. In that sense, IPC confers a ‘memory’ that could be encoded, for example, as matrix volume [122] or as the phosphorylated state of matrix K⁺ channels.

Since opening of the MPTP is such a critical factor for reperfusion damage, it is reasonable to ask how it may be connected to the other mechanisms. Experiments using inhibitors of the MPTP suggested that reduced opening of the MPTP may indeed represent one of the major factors in preconditioning [24,166,167,223,403]. Inhibition of GSK-3β is a hypothetical upstream event leading to an increase in the threshold for MPTP opening [122]. But there are other plausible mechanisms for decreasing the open probability of the MPTP. As outlined in Section 5.2, opening of K⁺ channels in the mitochondrial inner membrane is expected to reduce Ca²⁺ accumulation as well as ROS production during the reperfusion phase, two important factors mediating MPT. Furthermore, there is some experimental evidence that both activation of the mKATP channel and activation of the BKCa channel confer cardioprotection by inhibiting the opening of the MPTP during reperfusion [24,207]. Thus, it appears possible that the MPTP is a downstream target of mitochondrial K⁺ channel opening. This would bring together the three important factors involved in preconditioning, K⁺ channels, ROS and the MPTP, in one coordinated adaptive response of the heart to transient ischemia or hypoxia. Furthermore, PKCe (activated by ROS during the trigger phase) has been reported to physically interact with VDAC1, a component of the MPTP, and it was shown that PKCe-mediated inhibition of the MPTP contributes to cardioprotection [68]. These findings create another link between preconditioning and downstream signaling to the MPTP. Considering the massive alterations in fatty acid metabolism after ischemia and the universal inhibitory effects of 5-HD, a fourth factor, adaptive changes in fatty acid metabolism, may also be involved in the complex
sequence of events leading to cardioprotection. In this context it is interesting to note that acetyl-CoA carboxylase (see Fig. 5) is one of the downstream targets of GSK-3β ([419]).

Surprisingly, Halestrap et al. obtained evidence that a relatively short period of ischemia is sufficient to produce opening of the MPTP [406,421,423], and they concluded that the MPTP was able to close again after reoxygenation [223]. Hausenloy et al. [40] showed that the beneficial effects of IPC were abolished when either a MPTP blocker or a ROS scavenger was present during the trigger phase. They hypothesized that it was in fact the transient opening of the MPTP during the triggering phase and the concomitant release of ROS that induced the cardioprotective effect. They speculate that the preconditioning stimulus may induce transient MPTP opening by mediating uncoupling.

Javadov et al. [223] measured opening of the MPTP with the [3H]DOG entrapment technique [403] and concluded from their study that the blockers of MPTP opening were less effective than IPC in preventing MPTP opening. This important conclusion, and also the results of Hausenloy et al. [40] are consistent with the hypothesis that the primary event may be release of ROS after the brief trigger ischemia, followed by activation of PKC. The subsequent adaptive response, which involves K⁺ channels, matrix swelling and perhaps also transient opening of the MPTP or other mechanisms, inhibits MPTP opening after the index ischemia (see Section 5.2). Thus, assuming that transient opening of the MPTP during the trigger phase does in fact take place, it remains to be determined whether this opening is elicited by uncoupling [40], by ROS (Fig. 5) or by some other mechanism.

In conclusion, the results summarized in this review suggest that short periods of ischemia may activate an endogenous cardioprotective program that reduces the cell injury during subsequent periods of ischemia for several hours, or even for days, if the second window of protection is considered [3] (which has not been discussed here). Many factors are involved in this protective program, including activation of sK_ATP channels, activation of K⁺ channels in the mitochondrial inner membrane (possibly including mK_ATP channels), changes in fatty acid metabolism, release of ROS and opening the MPTP. Although inhibiting one of these factors may impair preconditioning, probably only the combination of these factors improves the resistance of the heart to further ischemic injury. Furthermore, recent findings suggest that regulation of cell metabolism and the regulation of cell survival/apoptosis may be functionally interconnected [80,416,424–428]. Thus, by modulating the MPTP and various other mitochondrial targets [415,416,420,429,430] the signal transduction pathways activated during preconditioning may enhance cell survival and antagonize apoptosis.

9. Summary and conclusions

9.1. Do mitochondrial K_ATP channels exist?

Ischemic or pharmacological preconditioning initiates a complex signaling cascade which extends the duration of ischemia that cells can tolerate without activating the cell death pathway upon reperfusion (Section 2). Mitochondrial K_ATP channels have been implicated in most studies of preconditioning, but the evidence for their structure and functional properties, or even their existence, has remained inconclusive. Several approaches have been used to characterize mK_ATP channels: (i) Single-channel recording in mitoplasts, (ii) partial purification of the mK_ATP channel protein and reconstitution into liposomes or lipid bilayers, (iii) measurement of light scattering of isolated mitochondria, (iv) measurement of flavoprotein fluorescence, and (v) measurement of ΔΨMIT with fluorescent dyes. None of the approaches, by itself, provided sufficient evidence to prove the existence of the channels (Section 3). It might be argued that, collectively, the studies on the properties of mK_ATP channels and their role in preconditioning make their existence more likely. However, the different aspects of the channels studied do not provide a unifying picture, and many of the basic characteristics of the putative mK_ATP channels, such as single-channel properties, ion selectivity, polarity of insertion into the membrane, sensitivity to inhibition by ATP, and structural similarities with sK_ATP channels or the MPTP remain unclear (Section 3).

The pharmacological approach has been extensively used to prove the existence of a distinct population of mK_ATP channels that shares some essential properties with sK_ATP channels (Section 4). Diazoxide, the most widely used preconditioning drug, is often assumed to activate selectively mK_ATP channels, but at preconditioning concentrations it exerts well-documented other actions, such as inhibition of SDH and protonophoric uncoupling, which independently confer cardioprotection. These actions also independently increase flavoprotein fluorescence, which has been used as an assay of mK_ATP channel activity in isolated myocytes. Thus diazoxide, and other mK_ATP channel openers (Section 4.1), have alternative targets, which limits their usefulness in identifying mK_ATP channels. The substituted fatty acid 5-HD is often assumed to be a selective mK_ATP channel inhibitor. However, 5-HD can be activated to 5-HD-CoA and metabolized in mitochondria, and one of its metabolic intermediates can inhibit fatty acid metabolism. Both 5-HD and the other presumed blocker of mK_ATP channels, glibenclamide, have multiple other actions that invalidate their use as selective blockers to identify the mechanisms underlying preconditioning (Section 4.2).

In conclusion, the hypothesis of mK_ATP channels as the initial trigger or as the end effector of preconditioning (or both) has been extremely fruitful and stimulated many sophisticated studies, the results of which remain valid. However, 14 years after the first description of mK_ATP channels [124] and 8 years after the proposal that mK_ATP channels may play a role in preconditioning [94,95], the existence of mK_ATP channels is still an open question, and it turned out that it is nearly impossible to prove the existence of a channel in the absence of selective activators or blockers, in particular if the gene coding for the channel is not known. Should we then
“throw away the ladder after we have climbed up upon it” [431]? We do not think so. It is not at all our intention to argue that $K^+$-selective channels with high sensitivity to ATP (these are the defining properties of $K_{ATP}$ channels) do not exist in the mitochondrial inner membrane. We just do not have sufficient experimental evidence either for or against, and the targets of diazoxide and 5-HD, very efficient modulators of preconditioning, remain unclear.

Several recent studies on preconditioning discuss the role of the putative $mK_{ATP}$ channels and the possible mechanisms of action of 5-HD and diazoxide as unresolved issues. We are therefore inclined to think that it may be time to end the obligatory ‘liaison’ between preconditioning and $mK_{ATP}$ channels. On one hand, it would be useful to devise further experiments on the mechanisms of cardioprotection that do not rely on the assumption that diazoxide opens and 5-HD blocks $mK_{ATP}$ channels. On the other hand, more direct evidence, independent of preconditioning, should be sought to prove or disprove the existence of $mK_{ATP}$ channels. Hopefully, further single-channel recordings in mitoplasts prepared from isolated cardiomyocytes will provide the answer.

9.2. An integrative view of preconditioning

Studies of ischemic preconditioning have helped to reveal a general adaptive response of the cells to metabolic stress. A brief period of ischemia represents a strong metabolic stimulus and elicits a coordinated response that makes the cardiomyocytes more resistant to injury during subsequent periods of ischemia. In fact, the adaptive response to short-term perturbation is slightly overcompensatory, and the overcompensated state is sustained for more than 1 h, representing the “memory” of preconditioning. We propose that at least five factors are involved in the response of the heart to metabolic stress: (i) opening of surface $K_{ATP}$ channels (Sections 6 and 7), (ii) release of reactive oxygen species (Section 8.1), (iii) opening of $K^+$ channels in the mitochondrial inner membrane (Section 5.2), (iv) changes in fatty acid metabolism (Section 8.2), and (v) modulation of the mitochondrial permeability transition pore (Section 8.3).

The studies with Kir6.2 knockout mice have clearly shown that $sK_{ATP}$ channels contribute to the cardioprotective effect of preconditioning. The $sK_{ATP}$ channel is a complex metabolic sensor that is integrated in a metabolic network that regulates energy transfer from mitochondria to the contractile proteins and to the surface membrane. This metabolic network, which includes creatine kinase, adenylate kinase and several metabolic intermediates, has an important function in adapting action potential duration, preventing mitochondrial Ca$^{2+}$ overload and regulating cellular energy demand. $sK_{ATP}$ channels play only a minor role during normal cardiac function but become very important during disturbances of energetic homeostasis such as hypoxia, ischemia and reperfusion (Sections 6 and 7).

There is general agreement that ROS scavengers applied during the trigger phase abolish preconditioning, and release of ROS is a strong candidate for the primary event that elicits the complex adaptive response of preconditioning (Section 8.1). The main sources of ROS are probably complexes I and III of the electron transport chain, but the mechanisms that cause the generation of ROS during the trigger phase are not yet clear. We hypothesize that ROS are released during reperfusion after the brief initial trigger period(s) of ischemia and then activate the cascade of events that ultimately leads to cardioprotection (Sections 5.2 and 8.4).

The mitochondrial inner membrane probably contains at least two or three $K^+$-selective channels: Ca$^{2+}$-activated $K^+$ channels ($mK_Ca$), voltage-activated $K^+$ channels ($mK_V$) and, perhaps, ATP-sensitive $K^+$ channels ($mK_{ATP}$). These channels may be activated via PKC, PKA and possibly other kinases during the trigger phase of preconditioning, during ischemia and during reperfusion. The opening of these channels is expected to produce mild uncoupling, depolarization and matrix swelling, depending on the metabolic state of the mitochondria. The combination of these effects is likely to reduce the production of ROS, especially during reperfusion, and this may be one of the major mechanisms responsible for preconditioning (Section 5.2). Thus, $K^+$ channels in the mitochondrial inner membrane are likely to be mediators of cardioprotection. Considering the massive alterations in fatty acid metabolism after ischemia and the universal inhibitory effects of the substituted fatty acid 5-HD, the fourth factor, adaptive changes in fatty acid metabolism, may also be involved in the complex sequence of events leading to cardioprotection (Section 8.2).

The mitochondrial permeability transition pore appears to be one of the key “end effectors” of preconditioning. Many studies have shown that inhibition of the MPTP during reperfusion confers cardioprotection. Various preconditioning signals appear to converge on the MPTP and prevent its opening, and initiation of cell death, in response to matrix Ca$^{2+}$ overload and oxidative stress. Indeed, receptor- or drug-induced activation of various signal kinases, such as PKA, PKC$\epsilon$, PI3K, and survival kinases such as Akt and ERKs, all implicated in cardioprotection, may effect protection by preventing/delaying opening of the MPTP. Furthermore, brief episodes of ischemia or various cardioprotective drugs may trigger preconditioning by transiently opening, directly or indirectly via ROS, the MPTP. While the latter hypothesis requires further testing, it is clear that regulation of the open probability of the MPTP, the fifth factor, plays a decisive role in preconditioning (Section 8.3).

Finally, we conclude that preconditioning activates a cellular survival program that requires the integration of several processes (Section 8.4) including opening of surface $K_{ATP}$ channels, regulation of fatty acid metabolism, ROS production, regulation of the mitochondrial permeability transition and opening of $K^+$ channels in the mitochondrial inner membrane.
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