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Review Article

The Slo(w) path to identifying the mitochondrial channels responsible for ischemic protection

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Mitochondria play an important role in tissue ischemia and reperfusion (IR) injury, with energetic failure and the opening of the mitochondrial permeability transition pore being the major causes of IR-induced cell death. Thus, mitochondria are an appropriate focus for strategies to protect against IR injury. Two widely studied paradigms of IR protection, particularly in the field of cardiac IR, are ischemic preconditioning (IPC) and volatile anesthetic preconditioning (APC). While the molecular mechanisms recruited by these protective paradigms are not fully elucidated, a commonality is the involvement of mitochondrial K^+ channel opening. In the case of IPC, research has focused on a mitochondrial ATP-sensitive K^+ channel (mitoK_{ATP}), but, despite recent progress, the molecular identity of this channel remains a subject of contention. In the case of APC, early research suggested the existence of a mitochondrial large-conductance K^+ (BK, big conductance of potassium) channel encoded by the *Kcnma1* gene, although more recent work has shown that the channel that underlies APC is in fact encoded by *Kcnt2*. In this review, we discuss both the pharmacologic and genetic evidence for the existence and identity of mitochondrial K^+ channels, and the role of these channels both in IR protection and in regulating normal mitochondrial function.

Ischemia–reperfusion injury and protection

Ischemia, defined as the blockage of delivery of oxygen and nutrients to tissues, is a pathologic event that underlies some of the most prevalent causes of death in humans. Paradoxically reperfusion (i.e., the re-establishment of oxygen and nutrient delivery) is also a pathologic event. Taken together, these events comprise ischemia–reperfusion (IR) injury, the underlying cause of diverse conditions such as heart attack and stroke. The focus of this review is cardiac IR; in the US alone, there are 750 000 heart attacks a year, killing 116 000 people. In addition, over 300 000 patients undergo a 'scheduled' cardiac ischemic event when the heart is arrested and placed on bypass during open heart surgery [1]. Since cardiac IR injury is a major cause of mortality and morbidity, it is surprising that beyond reperfusion itself (e.g., thrombolysis or balloon angioplasty), there are virtually no drug therapies to acutely treat it [2,3].

The heart is an energetically demanding tissue, with the bulk of its ATP demand met by mitochondrial oxidative phosphorylation [4,5]. Upon ischemia, mitochondrial ATP synthesis halts, starving processes such as actin/myosin cross-bridge cycling and the maintenance of ion gradients by the Na^+/K^+-ATPase and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). In addition, glycolytic metabolism generates lactate, causing cellular acidosis which then activates the Na^+/H^+ exchanger and leads to a rise in intracellular Na^+ [6,7]. Na^+ export is driven by the Na^+/Ca^{2+} exchanger, leading to a rise in cytosolic Ca^{2+} [8], which is compounded by the ATP-starved SERCA pump [9,10]. A cytosolic Ca^{2+} overload ensues, with some Ca^{2+} entering the mitochondrion. However, these events alone are insufficient to trigger opening of the mitochondrial permeability transition (PT) pore, since acidic pH and a reduced pyridine nucleotide pool (NADH) maintain the PT pore in a closed state [11–13]. At

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reperfusion, further Ca^{2+} overload occurs [14,15], pH rebounds [16], and a burst of reactive oxygen species (ROS) generation occurs as metabolites accumulated during ischemia are rapidly oxidized [17]. This combination of Ca^{2+}, pH and ROS triggers opening of the PT pore, leading to cell death [18–25]. These events are summarized in Figure 1.

Given the universality of IR injury as a pathologic insult in biology, it should not be surprising that a diverse array of organisms [26–31] exhibit mechanisms to limit damage due to this insult. Among the best studied of such mechanisms is ischemic preconditioning (IPC), in which short periods of prior IR afford protection against subsequent IR injury. IPC is an example of hormesis (i.e., 'what doesn't kill you makes you stronger') and is clinically applicable in humans [32]. IPC affords protection in two phases: the first develops in minutes, lasts 2–3 h, and involves cell signaling cascades that terminate at mitochondria [4], as will be discussed here. A second protective phase develops in ∼24 h and lasts up to 72 h, requiring gene transcription and *de novo* protein synthesis [33], but will not be considered further. Of particular interest for this review, it is also known that halogenated volatile anesthetics (halothane, isoflurane, sevoflurane, and desflurane) can mimic the protection afforded by IPC, a process known as anesthetic preconditioning (APC) [34–36].

The centrality of mitochondria to IR pathology has driven the organelle to be a natural focus for research on IR protection. In this regard, a common mechanism believed to underlie several cardioprotective paradigms, including IPC and APC, is the opening of potassium channels in the mitochondrial inner membrane [37–42]. This review will focus on the evidence for existence and identity of these channels.

Mitochondrial K^+ homeostasis and discovery of mitochondrial K^+ channels

A note on nomenclature

Before discussing these channels in detail, nomenclature should be clarified. The gene encoding the mitochondrial ATP-sensitive K^+ (K_{ATP}) channel is unknown, and so here we use the term 'mitoK_{ATP}'. For other K^+ channels, where possible the International Union of Basic and Clinical Pharmacology (IUPHAR) nomenclature is used [43] (see the Abbreviations list); however, there are many alternative names commonly found in the literature, described here.

The term 'BK' was coined in 1984 when a 'big K^+' or large-conductance K^+ channel activated by Ca^{2+} was recorded by patch clamp [44]. In 1986, the *Drosophila* slowpoke (Slo) mutation was shown to abolish a Ca^{2+}-activated K^+ current [45], and subsequently, the '*Slo1*' gene was shown to be conserved among phyla. Hence, BK (also known as 'maxi-K') was the term used to describe the channel encoded by the *Slo1* gene. This gene is now known as *Kcnma1*, and the channel is known as as $K_{Ca}1.1$.

A related family of *Slo* genes has since been identified, which now includes *Slo1* (*Kcnma1*), *Slo2.1* (*Kcnt2*), *Slo2.2* (*Kcnt1*), and *Slo3* (*Kcnu*) [46–52]. *Kcnma1* and *Kcnu* encode Ca^{2+}-activated K^+ (K_{Ca}) channels (now known as $K_{Ca}1.1$ and $K_{Ca}5.1$, respectively). Lower organisms such as *Caenorhabditis elegans* have a single gene (termed '*Slo2*') encoding a K_{Ca} channel [53], and here, we use the name SLO2 to refer to this channel in *C. elegans*. In contrast, this gene has diverged into two paralogs in mammals, and somewhat confusingly, the mammalian channels are, in fact, Na^+-activated (K_{Na}) channels: the gene previously known as *Slo2.1* was thought to encode a channel termed $K_{Ca}4.2$ (also known as 'Slick'). This gene is now known as *Kcnt2* and encodes a channel known as $K_{Na}1.2$. The gene previously known as *Slo2.2* was thought to encode a channel termed $K_{Ca}4.1$ (also known as 'Slack'). This gene is now known as *Kcnt1* and encodes a channel known as $K_{Na}1.1$. The umbrella term '$K_{Na}1.x$' is used here to refer to both mammalian $K_{Na}1.2$ and $K_{Na}1.1$ channels. Additional naming complexity is also imparted due to the channels encoded by the *Slo* family genes having alternate splice variants that can heteromultimerize [46,47,50,54,55] (see Sections 'Mitochondrial $K_{Ca}1.1$ Channels and Mitochondrial $K_{Na}1.x$ Channels').

The general term 'K_{Ca}' refers to the small-conductance (SK) channels, the intermediate-conductance (IK) channels [56], and the channel encoded by *Slo1* (*Kcnma1*). Unfortunately, 'K_{Ca}' is also sometimes used to reference all channels encoded by the *Slo* gene family, even though it is now apparent that many of these are K_{Na} channels (see above).

In the mitochondrial field, the terms 'mitoBK' and 'mitoK_{Ca}' (and sometimes even 'mitoBK_{Ca}'!) have been used interchangeably. Wherever possible, we attempt to define these channels using the IUPHAR nomenclature. However, many studies have assigned channel names on the basis of pharmacology alone, prior to the

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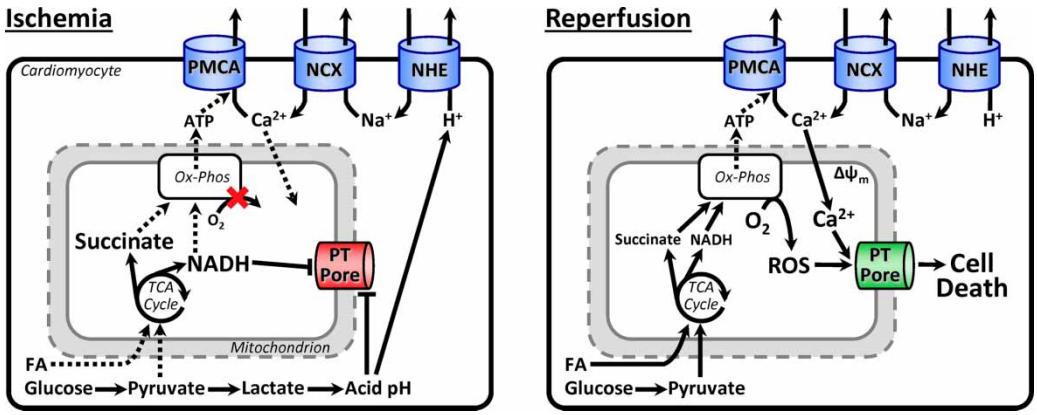

Figure 1. Schematic representation of pathologic events during ischemia and reperfusion.
Key events are listed below each figure and described in detail in the text (Section 'Ischemia–-Reperfusion Injury and Protection'). PMCA, plasma membrane Ca^{2+}-ATPase; NCX, Na^+/Ca^{2+} exchanger; NHE, Na^+/H^+ exchanger; FA, fatty acids; PT, permeability transition pore.

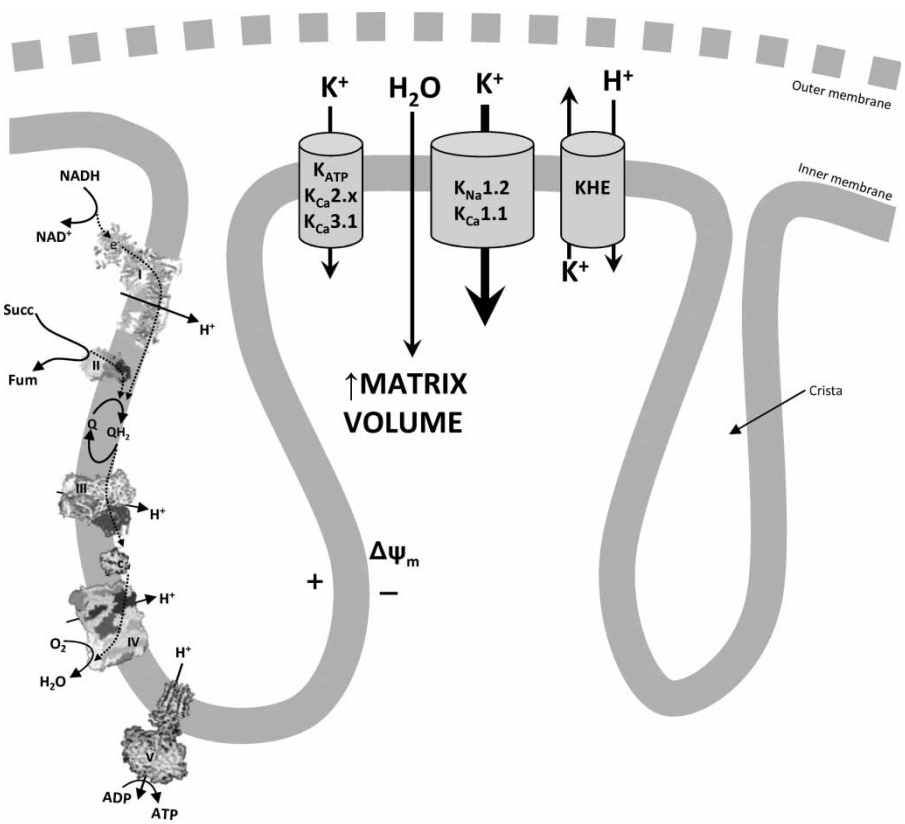

Figure 2. Mitochondrial K^+ cycle.
The mitochondrial inner membrane potential ($\Delta\psi_m$), which is generated by the respiratory chain (complexes I–IV [396,397], left), drives K^+ entry into the mitochondrial matrix through either small- or intermediate-conductance channels (e.g., K_{ATP}, $K_{Ca}2.x$, or $K_{Ca}3.1$) or large-conductance channels ($K_{Na}1.2$ or $K_{Ca}1.1$). This K^+ current is followed by osmotically obliged water, resulting in swelling of the matrix. K^+ is removed from the matrix through the KHE that also consumes $\Delta\psi_m$. The outer membrane is largely permeant to all solutes and hence is depicted as a dotted line.

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advent of molecular biological identification. Therefore, in such cases, we default to the nomenclature system used by the authors of these studies.

Mitochondrial K^+ channels

The mitochondrial inner membrane, while maintaining a tight barrier to proton permeability that is essential for its bioenergetic function, is also selectively permeable to numerous cations (K^+, Ca^{2+}, Mg^{2+}, and Na^+) [57–59] and anions (Cl^-, PO_4^-, nucleotide phosphates, and di- and tri-carboxylates) [60]. This selective permeability is under the control of membrane transporters, and here we focus on those ion channels mechanistically linked to IR protection — namely, the mitoK_{ATP} channel and the K^+ channels encoded by the *Slo* gene family. Other mitochondrial K^+ transport proteins [e.g., the K^+/H^+ exchanger (KHE) and voltage-gated K^+ channels] are reviewed extensively elsewhere [61–63].

Mitochondrial K^+ permeability has been studied since the early days of bioenergetics [64–66]. K^+ entry into mitochondria is accompanied by osmotically obliged water, resulting in swelling and a decreased refractive index [67], making mitochondrial volume (easily measured spectrophotometrically as the scattering of light by isolated mitochondrial suspensions) a useful surrogate measure of K^+ uptake [68–70]. Energetically driven swelling in K^+-containing buffers was initially attributed to an inherent K^+ permeability of the mitochondrial membrane [71,72], and subsequent studies identified an electrically neutral KHE supporting the existence of a mitochondrial K^+ cycle [73] (Figure 2). The fact that both K^+ influx and efflux consume the transmembrane H^+ gradient suggests the functional importance of the cycle, and it has been suggested that the cycle serves to regulate mitochondrial volume [74], which in turn may be an important regulator of respiratory function [75,76]. Alternatively, it has been proposed that mitochondrial K^+ homeostasis serves to regulate ROS production [77,78].

The first report of a *bona fide* mitochondrial K^+ channel by patch-clamp electrophysiology was in 1991 [79] and opened the way for identification of K^+ currents attributable to known K^+ channel families based on electrophysiological properties. Numerous K^+ channels have now been reported in mitochondria, including $K_V1.3$ in lymphocyte mitochondria [80], a K_{Ca} channel in liver [81] and fibroblast [82] mitochondria, and K^+_{ATP} in mitochondria from glioma [79] and cardiac ventricles [83]. Additional methods supporting mitochondrial K^+ channel identity include the following: (i) immunologic detection such as western blot [84] and fluorescent immunocytochemistry [85]. (ii) Mitochondrial fractionation and reconstitution of channels into liposomes [86]. (iii) Indirect measurement of mitochondrial K^+ uptake by fluorescent probes such as potassium binding fluorescent indicator [87] or fluorescent measurement of mitochondrial Tl^+ uptake as a surrogate for K^+ flux [68]. (iv) Genetic tagging of candidate K^+ channel proteins and their tracking to mitochondria within cells [88]. (v) Sensitivity of these measurements to a variety of pharmacologic agents that are known to act on particular classes of K^+ channel (see Sections 'Mitochondrial K_{ATP} Channel: Composition, Pharmacology, Regulation, Role in IR Protection; Mitochondrial $K_{Ca}2.x$ and $K_{Ca}3.1$ Channels: Composition, Pharmacology, Regulation, Role in IR Protection; and Mitochondrial $K_{Ca}1.1$ Channels and Mitochondrial $K_{Na}1.x$ Channels'). (vi) Generation of mice or cell lines with candidate mitochondrial K^+ channel genes deleted [89–91].

While these considerable efforts support the existence of *bona fide* K^+ channels in mitochondria, their molecular identities are still hotly debated. In particular, the mitoK_{ATP} channel is controversial [92–95]. This topic has been extensively reviewed elsewhere and so will be discussed only briefly here in the Section 'Mitochondrial K_{ATP} Channel: Composition, Pharmacology, Regulation, Role in IR Protection'. The identity of a mitochondrial large-conductance K^+ (BK) channel is also unclear [42,96–98] and is discussed in detail in Sections 'Mitochondrial $K_{Ca}2.x$ and $K_{Ca}3.1$ Channels: Composition, Pharmacology, Regulation, Role in IR Protection and Mitochondrial $K_{Ca}1.1$ Channels and Mitochondrial $K_{Na}1.x$ Channels'. For reference, a schematic of selected cardioprotective stimuli and their proposed target mitochondrial K^+ channels, along with inhibitors of such cardioprotection, is shown in Figure 3.

Mitochondrial K_{ATP} channel: composition, pharmacology, regulation, and role in IR protection

A study of the mitoK_{ATP} channel is incontrovertibly linked to the study of IR protection; much of the evidence for the existence of the channel comes from effects of channel-modulating drugs on cardiac IR injury, and much of the evidence for cardioprotection comes from the design and application of agents targeting putative

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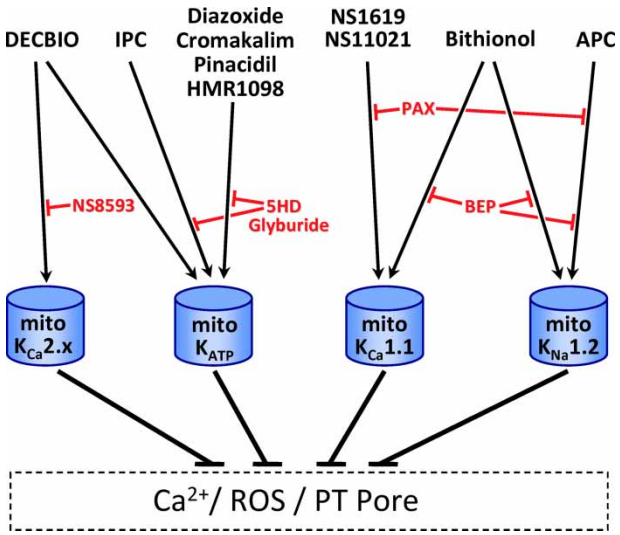

Figure 3. Cardioprotective stimuli, molecular targets, and inhibitors.

A subset of pharmacophores in Table 1 is known to confer cardioprotection. These are shown at the top of this figure, along with the cardioprotective stimuli of APC and IPC. Target mitochondrial K^+ channels are depicted below (blue), with known pharmacologic inhibitors of these protective paradigms in red. As detailed in the text, a combination of genetic and pharmacologic information has demonstrated that $K_{Ca}1.1$ and $K_{Na}1.2$ each participate in distinctly activated mechanisms of cardioprotection.

mitoK_{ATP} channels. Thus, research into this channel's composition or pharmacology is largely driven by its role in cardioprotection [99–101].

ATP-sensitive K^+ channels (K_{ATP}) have been detected in numerous cell membranes including plasma membrane [102], sarco/endoplasmic reticulum [103], mitochondria [79], and the nuclear envelope [104]. Generally, K_{ATP} channels are octamers composed of four 2-transmembrane inward-rectifying K^+ channel (KIR) subunits (KIR6.1/6.2), plus four 17-transmembrane sulfonylurea receptor (SUR) subunits (SUR1/2A/2B) [105]. The ventricular myocyte surface K_{ATP} channel comprises KIR6.2/SUR2A [106] and regulates both cell volume and action potential duration [107].

Initial observations suggested a role for surface K_{ATP} channels in IPC [108–110], with depressed contractility thought to be the mechanism of cardioprotection [107,111,112]. However, the discovery that K_{ATP} activators [diazoxide (DZX) [113], cromakalim [114], and aprikalim [115]] were capable of protecting noncontracting myocytes, in a manner blocked by K_{ATP} inhibitors [5-hydroxydecanoate (5-HD), glyburide, and HMR1098 [116–118]], suggested a protective mechanism independent of depressed contractility. The discovery of a mitochondrial K_{ATP} channel with sensitivity to DZX and cromakalim [79,83,119] provided a candidate mechanism, with further support provided by evidence that cardiac surface K_{ATP} channels are insensitive to DZX [120–123]. Subsequently, a channel with pharmacologic properties ascribed to mitoK_{ATP} was recognized as a key player in IPC signaling, despite an ongoing debate regarding the molecular identity of this channel. Weak evidence also exists for a potential role of a mitochondrial K_{Ca} channel in IPC [124,125] and will be discussed in Section 'Mitochondrial K_{Ca}1.1 Channels and Mitochondrial K_{Na}1.x Channels'.

Although global knockout mice exist for K_{ATP} channel subunits (i.e., KIR6.1/6.2 and SUR1/2), several complications preclude their use to study IPC. For example, *Kir6.1*$^{-/-}$ mice [126] exhibit a form of angina, and it is known that patients with unstable angina exhibit a preconditioned phenotype [127,128]. While *Kir6.2*$^{-/-}$ mice [91,121] exhibit blunting of protection by IPC, these mice also have impaired insulin secretion and mild glucose intolerance [91], and it is known that diabetes abrogates protection by IPC [129]. Similarly, both *Sur1*$^{-/-}$ and *Sur2*$^{-/-}$ mice exhibit glycemic disturbances and are endogenously protected against cardiac IR injury [130–132], precluding their use to study cardioprotective signaling. Furthermore, the *Sur1*$^{-/-}$ mouse was demonstrated to still express alternate splice variants [133], again confounding studies attempting to assign functions to the SUR1 protein. Owing to these confounds, assignment of a particular combination of KIR/SUR subunits as underlying IPC has not been possible to date. In addition, evidence favoring a mitoK_{ATP}

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Table 1 Commonly used pharmacologic agents in the fields of mitochondrial K^+ channel research and cardioprotection, for K_{ATP}, $K_{Ca}2.x$, $K_{Ca}1.1$, and $K_{Na}1.x$ channels

Channel	Actions	Drugs	EC_{50}/IC_{50}	Refs
K_{ATP}	Activators	Atpenin A5	10 nM	[39]
		Cromakalim	1 μM	[113,114,398]
		Diazoxide	10 μM	[113]
		Pinacidil	50 μM	[115,399,400]
	Inhibitors	Fluoxetine	2.4 μm	[68]
		Glyburide	50 μm	[113,119]
		5-HD	100 μM	[116]
		Quinine	100 μM	[119]
		HMR1098	100 μM	[119]
$K_{Ca}2.x$	Activator	DECBIO	3 μm	[180]
	Inhibitors	ChTx	50 nM	[191]
		Apamin	1 μM	[191]
		Fluoxetine	9 μM	[192]
		NS8593	10 μM	[180]
$K_{Ca}1.1$	Activators	Emodepside	14 nM	[258]
		Rottlerin	500 nM	[238]
		NS11021	500 nM	[242]
		NS004	10 μM	[401]
		NS1619	10 μM	[242]
		17-β Estradiol	30 μM	[313]
		Niflumic acid	33 μM	[309]
		Ethanol	20 mM	[315]
	Inhibitors	SloTx	1.5 nm	[402]
		IbTx	50 nM	[232]
		Charybdotoxin	200 nM	[403]
		Paxilline	1 μm	[42]
$K_{Na}1.x$	Activators	Niclosamide	2.9 μM	[317]
		loxapine	4.4 μM	[317]
		17-β Estradiol	10 μM	[314]
		Bithionol	10 μM	[310]
		Isoflurane	300 μM	[307]
		Niflumic acid	2.1 mM	[294]
	Inhibitors	Bepridil	500 nM	[310]
		Pax	1 μM	[97]
		Verapimil	100 μM	[291]
		Quinidine	100 μM	[308]
		Clofilium	109–331 μM	[290]

composition of the canonical KIR6/SUR proteins needs to be balanced against the discovery that the original antibodies used to identify these proteins in purified mitochondria recognize off-target proteins unrelated to K^+ channel function [134]. Furthermore, although a smaller 55 kDa splice variant of SUR2A has been reported in mitochondria [135], this was detected using custom antibodies which failed to detect the same 55 kDa band in similar samples from the same laboratory a year earlier [133]. A subsequent study [136] also suggested rather equivocal evidence for the existence of this 55 kDa band, and overall caution should be used in interpreting any immunologic evidence for a mitoK_{ATP} channel.

The pharmacology of mitoK_{ATP} is conserved in humans [137], rats [79], plants [138], amoeba [139], trypanosomes [140], and *C. elegans* [141], and remains the default method to assign a role for mitoK_{ATP} in IR protection. A catalog of K^+ channel pharmacophores applicable to mitochondrial research is given in Table 1. While many channel modulators are available, the two most commonly linked to mitoK_{ATP} are the channel-activating benzothiadiazine derivative DZX and the antagonist 5-HD [142]. We see that 10 μM DZX is a relatively specific mitoK_{ATP} agonist and mimics the protective effects of IPC [113,116], whereas 5-HD prevents both IPC- and DZX-mediated IR protection [143,144]. At concentrations >40 μM, DZX has several other mitochondrial effects (e.g., complex II inhibition and protonophoric activity [145,146]). The specificity of

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5-HD has also been questioned, since it undergoes β-oxidation to yield 5-HD-CoA and other derivatives [147,148]. However, this compound is also effective within 1 s in mitoK$_{ATP}$ assays, suggesting that such metabolism is irrelevant for its acute effects on the mitoK$_{ATP}$ [149]. Additional K$_{ATP}$ channel activators including cromakalim and pinacidil [150–153] (Table 1) have also been studied in the context of cardiac IR protection and are thought to elicit protective effects via the mitoK$_{ATP}$.

A common feature that has arisen in the field of mitoK$_{ATP}$ pharmacology is complex II of the mitochondrial respiratory chain [39,145,149,154–157]. In short, several compounds that open the mitoK$_{ATP}$ channel are known to be complex II inhibitors, and in turn many complex II inhibitors have been discovered to open the channel. Among these, the most potent is the complex II inhibitor atpenin A5 (AA5), which is an effective mitoK$_{ATP}$ activator at low nM concentrations and is cardioprotective in a manner blocked by 5-HD [39,157,158]. Additionally, a mitochondrial membrane fraction enriched in complex II, mitochondrial ATP-binding cassette protein 1, phosphate carrier, adenine nucleotide translocator, and ATP synthase was shown to have mitoK$_{ATP}$-like activity when reconstituted in lipid bilayers [86]. The exact nature of the relationship between complex II and the mitoK$_{ATP}$ is reviewed extensively elsewhere [159]. Similarly, an interaction between complex IV and a paxilline-sensitive K^+ channel has been reported in membranes isolated from brain mitochondria [160], although the identity of this channel is currently unclear.

Despite the assignment of IPC protection to a mitochondrial channel with K$_{ATP}$-like properties, the molecular identity of the channel remains unclear. Specifically, none of the KIR6.1/6.2 or SUR1/2A/2B proteins are known to contain mitochondrial targeting sequences [134,161–165]. Furthermore, although there are 14 KIR channel isoforms in mammals [105,166], the genetic model organism *C. elegans* contains only three such proteins (encoded by the *irk-1,2,3* genes) [167]. In *C. elegans* with ablation of all three *irk* genes, no alteration in protection by hypoxic preconditioning or baseline sensitivity to hypoxic injury was seen [88]. Furthermore, mitochondria from these worms exhibited K^+ channel activity with all of the pharmacologic properties of a K$_{ATP}$ channel [88]. These data suggest that mitoK$_{ATP}$ might not be a canonical KIR6/SUR channel.

Alternatively, another member of the *Kir* gene family, *Kir1.1* (also known as renal outer medullary K^+ channel, ROMK), has recently been proposed to encode a mitoK$_{ATP}$ channel [95]. Specifically, ROMK variant 2 (ROMK2) contains an N-terminal mitochondrial localization sequence, and although its endogenous expression could only be detected by reverse-transcriptase polymerase chain reaction (RT-PCR), overexpression of recombinant ROMK2 fused to an epitope tag allowed immunodetection of its co-localization with mitochondrial markers. Recently, specific ROMK2 activators have been reported [168–170], but it is yet to be determined if these molecules can elicit protection against IR injury or activate a mitochondrial K^+ flux. In addition, although a whole-body ROMK knockout exists [171,172], renal insufficiency and hypertension render this model unsuitable for cardiovascular studies such as IR injury, and as of the submission of the present study, a cardiac-specific ROMK knockout mouse has not been reported. Finally, the pharmacologic properties of the mitoK$_{ATP}$ (e.g., sensitivity to DZX ATP, phosphadityl inositol bisphosphate (PIP_2), and fluoxetine) do not match those reported for ROMK (see ref. [159]). It is also intriguing that a recent abstract [173] claims to have identified the mitoK$_{ATP}$ channel as a previously unknown protein (i.e., not ROMK). Hence, it seems prudent to keep an open mind as to whether ROMK2 is the *bona fide* mitoK$_{ATP}$ channel.

Mitochondrial K$_{Ca}$2.x and K$_{Ca}$3.1 channels: composition, pharmacology, regulation, and role in IR protection

There are four genes in the *Kcnn* family. *Kcnn1*, *Kcnn2*, and *Kcnn3*, respectively, code for the SK potassium channels with IUPHAR names K$_{Ca}$2.1, K$_{Ca}$2.2, and K$_{Ca}$2.3 [174], while the *Kcnn4* gene encodes an IK potassium (IK) channel termed K$_{Ca}$3.1 [43]. The K$_{Ca}$3.1 channel has not been implicated in IR protection; however, it does have a role in post-ischemic cardiac remodeling [175,176] and *Kcnn4*$^{-/-}$ mice exhibit more damage in ischemic stroke [177]. The K$_{Ca}$2.x channels are expressed in atrial cells but not in ventricular tissue [178]. Their activation by DECBIO is protective against cardiac IR injury [179,180], and this protection is blocked by the K$_{Ca}$2.x antagonist NS8593. More recently, it has been claimed, on the basis of immunologic evidence, that the K$_{Ca}$2.x channels responsible for this cardioprotection are mitochondrial, and of the K$_{Ca}$2.2 and K$_{Ca}$2.3 variety [180], although similarity between these SK proteins precludes identification of the exact subtype.

K$_{Ca}$2.x channels are nominally activated by sub-micromolar Ca^{2+}, co-ordinated by a calmodulin (CaM)-binding domain. Additional regulation of channel activity is also afforded by phosphorylation at the N- and C-termini [181–183]. These channels are also known to be activated by stimuli implicated in cardioprotection, such as

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11,12-epoxyeicosatrienoic acid (EET) and NO· [184,185]. However, both of these species can elicit protection via pleiotropic mechanisms [186,187], including other K_{Ca} channels [188–190]. The $K_{Ca}2.x$ channels are selectively blocked by apamin, and are also nonselectively blocked by charybdotoxin (ChTx) [191] and fluoxetine, which also target BK and K_{ATP} channels, respectively [157,192]. This overlapping pharmacology with BK and K_{ATP} channels should be taken into consideration when interpreting pharmacologic evidence for a mitochondrial or cardioprotective role of $K_{Ca}2.x$ channels. Ultimately, the use of *Kcnn1–4*$^{-/-}$ mice [177,193,194] may be informative regarding the contribution of these channels to IR protection.

Mitochondrial $K_{Ca}1.1$ channels

$K_{Ca}1.1$ channels — composition

The $K_{Ca}1.1$ channel consists of a tetramer of *Kcnma1* encoded pore-forming α subunits, each of which can be accompanied by a β subunit, with the entire complex also binding γ subunits. Each α subunit has seven transmembrane helices, a voltage sensor, β/γ interaction domains, and a large cytosolic region containing two RCK (regulation of conductance of K^+) domains which house the Ca^{2+} sensing 'bowl'. The exon 19–23 region of *Kcnma1* (between the RCK domains) can be alternatively spliced (Figure 4) [48,195–197], yielding isoforms termed 'zero' (no exons 19–23), 'e20' (IYF insert between 19 and 20) [198], 'e21/STREX' (59 AA insert in exon 21) [198–201], 'e22' (inclusion of exon 22) [198], 'Δe23' (loss of exon 23) [202,203], and 'DEC' (C-terminal splice variant) [204]. Notably, the DEC variant has been suggested to impart mitochondrial localization [204]; however, this moiety has also been observed to increase surface $K_{Ca}1.1$ expression in combination with a β4 subunit [205,206].

There are four genes encoding $K_{Ca}1.1$ β subunits (*Kcnmb1–4*) [207,208] and their expression is tissue-specific [208,209]. These proteins interact with $K_{Ca}1.1$-α, altering activity and drug sensitivity [209–214]. β1 and β2 both increase Ca^{2+} sensitivity [213–216], and β1 also slows activation/inactivation kinetics [213,217] and affects cellular localization [215]. β2 and some splice variants of β3 also contain globular N-terminal domains, which confer rapid 'N-type inactivation' to $K_{Ca}1.1$ currents [211,214,218–222], whereas β4 down-regulates channel activity [223]. Mitochondrial localization of the β subunits, particularly β1 [189], β3 [82], and β4 [189], has been reported; however, these reports are largely informed by co-immunoprecipitation of the β subunits with the α subunit from mitochondrial-enriched tissue preparations. Currently, there are no data demonstrating that the β subunits complex with the α subunits in mitochondria and affect the channels' pharmacologic or electrophysiologic properties. $K_{Ca}1.1$ γ subunits are single transmembrane leucine-rich repeat proteins encoded by the *Lrrc26* (γ1), *Lrr52* (γ2), *Lrr55* (γ3), and *Lrr38* (γ4) genes. The γ subunit interaction with the extracellular face of $K_{Ca}1.1$-α lowers channel voltage sensitivity [224,225]. Although γ subunits are diversely expressed, to date none have been detected in the heart [226].

Knockout mice exist for the genes encoding several components of the $K_{Ca}1.1$ channel, including the *Kcmna1* [90,227], *Kcnmb1* [220], and *Kcnmb4* [228]. These mice exhibit a variety of phenotypes including spontaneous death, motor dysfunction, circadian rhythm disruption, and vasoconstriction, although this has not precluded their use in studying the role of $K_{Ca}1.1$ in cardioprotection (see below). Functional insight has also been afforded by $K_{Ca}1.1$ channel crystal structures [229,230].

$K_{Ca}1.1$ channels — pharmacology and regulation

There are no drugs that distinguish $K_{Ca}1.1$-α splice variants, although β/γ subunits have been shown to affect $K_{Ca}1.1$ pharmacology. The peptide toxins ChTx, iberotoxin (IbTx), and slotoxin (SloTx) all occlude the pore on the outer face of the channel [231–233] and are useful for measuring surface $K_{Ca}1.1$ function, but their membrane impermeability renders them unsuitable for probing intracellular $K_{Ca}1.1$ activity. Sensitivity to ChTx is increased 20-fold by β1 [231], whereas sensitivity to SloTx is decreased by β1 or β4 [234], and inhibition by ChTx or IbTx is lost in the $K_{Ca}1.1$-α/β4 composition [235,236]. Although the molecule rottlerin (also known as mallotoxin, historically thought to be a PKC inhibitor [237]) is known to activate $K_{Ca}1.1$ [238], γ1 subunit-containing channels are resistant to such activation [239,240]. The small-molecule paxilline is reportedly a membrane-permeable $K_{Ca}1.1$ blocker, but its specificity has been questioned by the reported efficacy in *Kcnma1*$^{-/-}$ mice [97]. The neurosearch (NS) class of compounds (NS004, NS1619, and NS11021) was developed as $K_{Ca}1.1$-specific activators [241,242] and is cardioprotective (Figure 2 and Table 1) [96,243–245], but also exhibits multiple $K_{Ca}1.1$-independent effects [246–253]. This includes inhibition of SERCA [254], L-type Ca^{2+} channels [246], Ca^{2+}-activated Cl^- and Na^+ channels [247], and Ca_V channels [248,255,256]. In addition,

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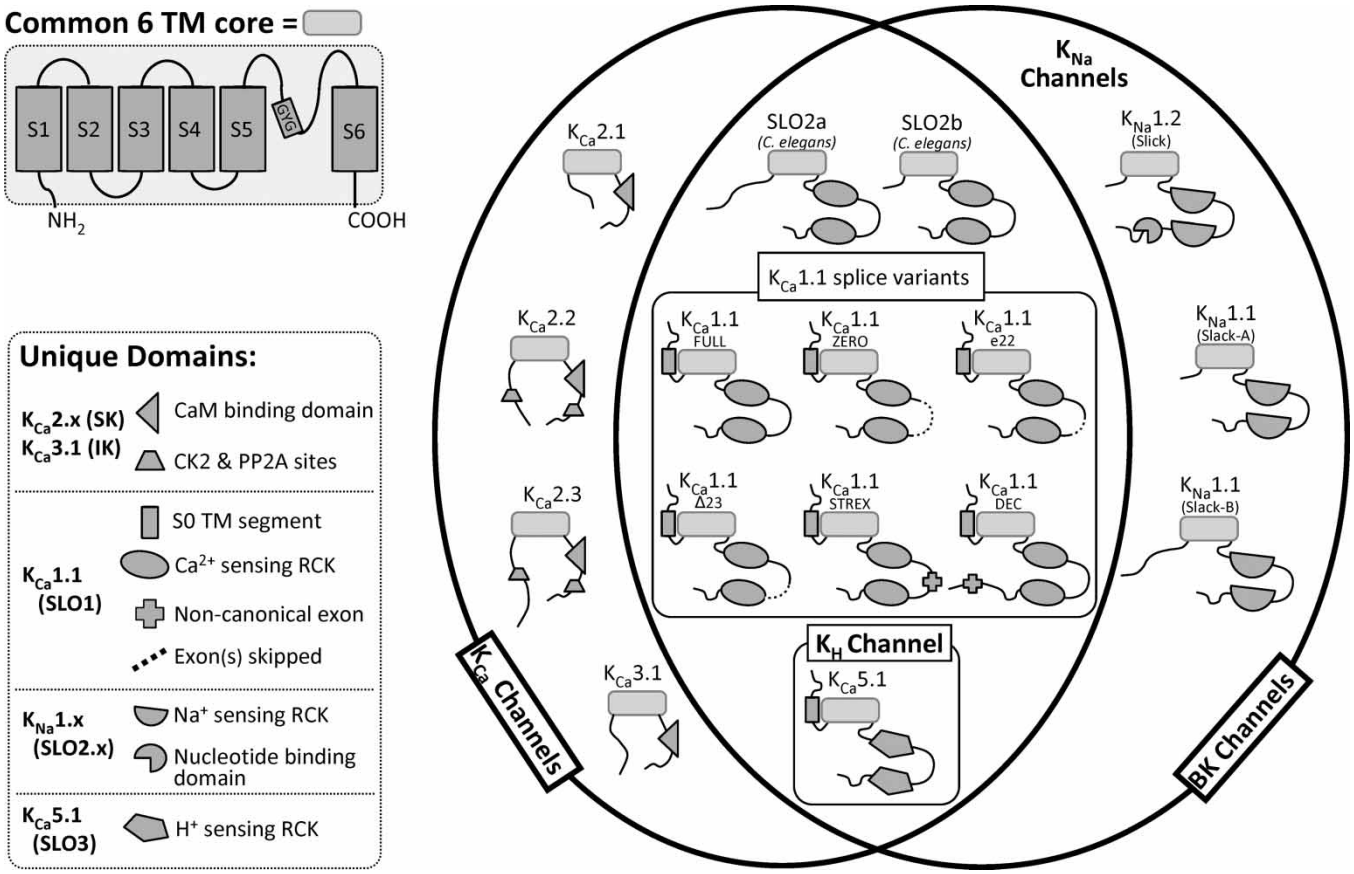

Figure 4. Venn diagram representing the various K^+ channel channels, isoforms, and their subgroupings.

The 6-transmembrane (6TM) pore-forming core is represented as a rounded rectangle with the individual N- and C-termini and their corresponding unique exons/domains illustrated as darker shaded shapes (see key). As only gross structural motifs are depicted in this figure, specific differences in amino acid sequence (i.e., differences in charged residues in S4 between $K_{Ca}1.1$ and $K_{Na}1.x$ or unique sequences in $K_{Na}1.1$ and $K_{Na}1.2$ RCK domains) are not represented.

these drugs activate $K_V7.4$ and nAchR α7, and are known to have multiple effects on mitochondrial function [98,252,257]. Finally, emodepside has been reported to activate $K_{Ca}1.1$ at nM concentrations [258,259]. Overall consensus is that peptide-based $K_{Ca}1.1$ modulators are specific but of limited *in situ* utility, whereas current small-molecule $K_{Ca}1.1$ modulators are less specific but more useful in a variety of cell, organ, or *in vivo* settings.

In addition to direct pharmacology, many signaling pathways are known to endogenously regulate $K_{Ca}1.1$ channels in a cellular context. These include arachidonic acid metabolism (i.e., EETs) [189,260], $NO^{\cdot}$ metabolism [261,262], pH [263], Zn^{2+} homeostasis [264], phosphorylation by PKA, PKG, PKC, and CaMKII [265–268], and palmitoylation and myristoylation [269–271]. Unfortunately, given the enormous scope of this subject, full discussion of these signaling pathways and their context in IR injury and protection is not possible in the current review. Finally, given that $K_{Ca}1.1$ is a K_{Ca} channel, it should be noted that modulation of intracellular Ca^{2+} by other Ca^{2+} channels (e.g., ryanodine and IP_3 receptors) can also affect its activity [272–275].

Mitochondrial $K_{Ca}1.1$ channels — role in IR protection

The first report of a mitochondrial large-conductance (295pS) K^+ channel employing patch clamp of glial cell mitochondrial inner membranes [81] showed that the channel was activated by Ca^{2+} (0.1–1 μM) and voltage, and blocked by ChTx. A large-conductance K^+ (BK) channel opened by NS1619 was also found in isolated liver mitochondria, and this compound was protective in a rabbit heart model of IR injury, in a manner blocked by paxilline [42]. Further work confirmed the cardioprotective nature of the NS compounds and also

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reported on a potential role for a mitochondrial BK channel in cardioprotection by volatile APC [74,97,242]. Specifically, the $K_{Ca}1.1$ antagonist IbTx was reported to block volatile anesthetic or NS protection in a model of ischemic postconditioning [276]. These studies assigned a variety of names to the mitochondrial BK channel including BK, K_{Ca}, and BK_{Ca} (see Section 'Mitochondrial K^+ Homeostasis and Discovery of Mitochondrial K^+ Channels'), although a specific gene or protein name was conspicuously absent. Notably, cardioprotection induced by the $K_{Ca}1.1$-activating NS compounds was not blocked by the mitoK_{ATP} antagonist 5-HD [125]. In addition, the mitochondrial BK channel itself was found to be insensitive to DZX and 5-HD [277], suggesting that this channel is distinct from mitoK_{ATP}. Later, immunologic studies documented $K_{Ca}1.1$ expression in the heart [196,198,207], and it was found that $K_{Ca}1.1$ channels are not expressed at the myocyte plasma membrane or sarcoplasmic reticulum membrane [42,277]. Furthermore, $K_{Ca}1.1$ was found specifically in mitochondrial membranes [42,81,204,278,279], including the presence of the β1 subunit [41,42,278,280,281]. Together, these studies suggested the existence of a *bona fide* $K_{Ca}1.1$ channel in the mitochondrial inner membrane, with a proposed role in mediating the cardioprotective effects of APC.

Despite these early studies, the case for a role of $K_{Ca}1.1$ in cardioprotection by APC has previously been challenged by our finding that APC protection was intact in *Kcnma1*$^{-/-}$ hearts [97]. Thus, $K_{Ca}1.1$ is dispensable for APC. Furthermore, as detailed above, the specificity of the NS compounds for $K_{Ca}1.1$ has been repeatedly questioned [119,246–253,257]. For example, the PKA inhibitor H-89 blocks APC protection [282], but does not block protection afforded by NS1619 [41], thus suggesting that NS compounds and APC protect via different mechanisms. In general, the NS compounds are unsuitable for drawing conclusions about the molecular identity of channels that underlie APC.

This concept is further illustrated by the finding that while IR protection by NS compounds was absent from *Kcnma1*$^{-/-}$ hearts, it was still present in *Kcnma1*$^{-/-}$ cardiomyocytes. This suggests that a $K_{Ca}1.1$-independent side effect of the NS compounds was responsible for their protective effects in the isolated cell system [98], but for poorly understood reasons this $K_{Ca}1.1$-independent side effect was not able to be recruited for protective benefit in the intact heart. Further experimentation revealed a role for $K_{Ca}1.1$ channels within intrinsic cardiac neurons (where $K_{Ca}1.1$ is known to be expressed [283,284]) in mediating the protective effects of NS compounds in the intact heart [98]. Overall, it is suggested that APC cardioprotection does not require $K_{Ca}1.1$, whereas NS cardioprotection requires $K_{Ca}1.1$ in a noncardiomyocyte cell (probably cardiac neurons). It should be noted that our data do not preclude the possibility that a mitochondrially localized $K_{Ca}1.1$ channel exists and may indeed be a viable drug target to induce cardioprotection. However, such a channel has no role in APC and cannot be inferred from the use of NS compounds.

Finally, although the use of nonspecific K_{Ca} channel inhibitors has led to suggestions of a potential role for mitochondrial $K_{Ca}1.1$ channel in IPC [124,125], such a case is refuted by our finding that protection induced by IPC was completely intact in *Kcnma1*$^{-/-}$ hearts [98]. Thus, without a defined role in IPC or APC, any potential cardioprotective effects of a mitochondrial $K_{Ca}1.1$ channel are limited to specific agonists that are yet to be discovered.

Mitochondrial $K_{Na}1.x$ channels

$K_{Na}1.x$ channels — composition

The $K_{Na}1.x$ channels consist of tetramers of pore-forming α subunits encoded by either *Kcnt1* or *Kcnt2*. These genes are classified as being in the *Slo* gene family, despite having only 7% homology to *Kcnma1*. $K_{Na}1.x$ channels lack the S0 domain of $K_{Ca}1.1$ and are therefore unable to interact with β subunits [50]. They also lack the voltage-sensing positive residues in transmembrane S4. $K_{Na}1.x$ and $K_{Ca}1.1$ also differ in their cytosolic domains, with $K_{Na}1.x$ RCK domains activated by Na^+ and Cl^- and inhibited by Ca^{2+} (Figure 4) [285]. The $K_{Na}1.1$ paralog has five splice variants (termed Slack-A, Slack Ax2, Slack B, Slack Bx2, and Slack M [286]) in the cytosolic N-terminal region, of which two have been characterized: Slack-B is the canonical $K_{Na}1.1$ channel, whereas Slack-A produces a channel with properties similar to $K_{Na}1.2$ [49,55,286]. While no splice variant of $K_{Na}1.2$ has been discovered, $K_{Na}1.2$ can heteromultimerize with Slack-B [55] or with $K_{Ca}1.1$, to produce channels with novel characteristics [50]. The $K_{Na}1.x$ channels have been mainly studied in the brain where they are highly expressed, and currently, only $K_{Na}1.2$ has been detected in the heart [51,89,286,287]. This is consistent with early observations of surface K_{Na} channels in the heart [288].

The $K_{Na}1.2$ channel is unique among channels encoded by the *Slo* gene family, in that it has a nucleotide-binding domain on the C-terminus (Figure 4). ATP binding to this domain inhibits the channel in rodent cells

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[49], but activates in human and frog cells [287,289–297]. Meanwhile, NAD^+ and $NADP^+$ both activate $K_{Na}1.2$ presumably via this nucleotide-binding domain [298], but it should be noted that pyridine nucleotides can also act on K_{ATP} [299] and K_{Ca} channels [300,301], so this property cannot be used to distinguish these channels. Interestingly, in neurons of both humans and rats, $K_{Na}1.2$ transcription is regulated by nuclear factor kappa B (NF-κB) activation [302], which itself is activated by IPC and APC [303–306]. Knockout mice for *Kcnt1*, *Kcnt2*, and a double knockout are available and have been used to determine the role of these channels in cardioprotection (see below) [89,307].

$K_{Na}1.x$ channels — pharmacology and regulation

$K_{Na}1.x$ channel pharmacology is largely informed by $K_{Na}1.1$, although several of these drugs are also known to affect the lone *C. elegans* SLO2 channel [97], and thus, not surprisingly, most $K_{Na}1.x$ drugs exhibit similar effects on both mammalian paralogs. There are numerous drugs that nonspecifically inhibit both $K_{Ca}1.1$ and $K_{Na}1.x$ channels, including paxilline, verapamil, and bepridil [308,309]. Similarly, several drugs can nonspecifically activate both $K_{Ca}1.1$ and $K_{Na}1.x$ channels: bithionol [310–312], 17β-estradiol [313,314], and nonsteroidal anti-inflammatory fenmates such as niflumic acid [294]. In addition, several drugs that target other K^+ channels do not affect $K_{Na}1.x$ channels, including (target) dendrotoxin (K_V), apamin ($K_{Ca}2.x$), glibenclamide (K_{ATP}), or DZX (K_{ATP}). This can help in ruling out a role for K_{Na} channels in any particular phenomenon [49,50].

In terms of distinguishing $K_{Ca}1.1$ from $K_{Na}1.x$, ChTx and IbTx have already been described above as $K_{Ca}1.1$-specific inhibitors, and NS1619 and EtOH as $K_{Ca}1.1$ activators; none of these agents affect $K_{Na}1.x$ [315]. There are also inhibitors of $K_{Na}1.x$ including clofilium and quinidine [290,308,309,316] and activators of $K_{Na}1.x$ including loxapine and niclosamide (see Table 1) [317]. None of these agents have an impact on $K_{Ca}1.1$. Unfortunately, many of these drugs also affect other channels, including $K_{Ca}5.1$ (also known as SLO3, encoded by *Kcnu*) [318], K_V11 (human ether-a-go-go related gene) [319], K_V10 (human ether-a-go-go potassium channel) [320], $K_V1.5$ [321], K_V7 (*Kcnq*) [322], and K_V4 (*Kcne*) [323] channels. As such, the use of pharmacologic agents to assign distinct functions to $K_{Na}1.x$ channels is susceptible to a multitude of off-target effects.

In addition to pharmacologic regulation, several signaling pathways are known to regulate $K_{Na}1.x$ channels either directly or at the transcriptional level. NAD^+ regulates both $K_{Na}1.2$ [298] and $K_{Na}1.1$, resulting in activation and a lower EC_{50} for Na^+. In the brain, $K_{Na}1.2$ channels are inhibited by PKC, G_α, G_q, M1, and mGluR1 receptor signaling pathways, whereas $K_{Na}1.1$ channels are activated by these same pathways [55].

$K_{Na}1.x$ channels — role in mitochondria and cardioprotection

In isolated cardiac mitochondria, functional assays (Tl^+ flux) have demonstrated a channel with BK-like pharmacologic sensitivity (i.e., activation by bithionol or isoflurane and inhibition by bepridil) that is absent from mitochondria from *Kcnt2*$^{-/-}$ mice [307]. This channel was still present in *Kcnt1*$^{-/-}$ mice and absent from *Kcnt1*/*Kcnt2* double knockouts, supporting the existence of a $K_{Na}1.2$ channel in heart mitochondria [307]. To date, immunologic and other approaches have not yielded solid evidence for the mitochondrial $K_{Na}1.2$ channel, primarily due to issues of antibody specificity (unpublished observations), although this is a problem not unique to $K_{Na}1.2$ (see discussion above on mitoK_{ATP} and $K_{Ca}1.1$ channels). Electrophysiology (patch clamp) studies on mitoplasts from wild-type and *Kcnt2*$^{-/-}$ mice are currently underway in our laboratory.

Both *Kcnt2*$^{-/-}$and *Kcnt1*$^{-/-}$ mice retain cardioprotection by IPC and DZX, consistent with the action of a mitoK_{ATP} channel and not a $K_{Na}1.x$ channel in such protection. In addition, the *Kcnt1*$^{-/-}$ heart was protected by APC (via isoflurane). However, in *Kcnt2*$^{-/-}$ and in *Kcnt1*/*Kcnt2* double knockout hearts, no protection by APC was observed [307]. These data suggest an absolute requirement of $K_{Na}1.2$ for the protective effects of APC. Furthermore, the $K_{Na}1.x$ activator bithionol was found to be protective when delivered exogenously, supporting that opening of $K_{Na}1.x$ alone is sufficient to confer IR protection.

The single *C. elegans* SLO2 channel is also required for hypoxic protection. Specifically, protection against anoxia–reoxygenation injury by the volatile anesthetic isoflurane was lost in *Slo2*$^{-/-}$ worms. In addition, mitochondria from *Slo2*$^{-/-}$ worms lacked a BK-like channel activity seen in wild-type mitochondria (i.e., activated by bithionol or isoflurane, blocked by bepridil, and insensitive to IbTx) [97]. Taken together, these knockout organism experiments demonstrate that a channel with BK-like activity in the mitochondrion (SLO2 in worms and $K_{Na}1.2$ in mammals) is a conserved mechanism for protection against IR injury triggered by APC.

In the context of ion homeostasis in IR injury and cardioprotection, there are clear reasons for hypothesizing that both Na^+-activated and Ca^{2+}-activated mitochondrial channels (i.e., $K_{Na}1.2$ and $K_{Ca}1.1$, respectively) may

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be opened by the high prevailing concentrations of Na^+ or Ca^{2+} during ischemia (see Section 'Ischemia–Reperfusion Injury and Protection') [51,324–326]. However, evidence for the opening of these channels in baseline IR injury alone is lacking, and blockers of these channels do not exacerbate IR injury [92,307]. In addition, how the levels of Na^+ and Ca^{2+} in the heart differ in ischemia following either IPC or APC is poorly understood. There is currently no rationale for the hypothesis that these channels would open in response to their natural ligands (Na^+ or Ca^{2+}) under cardioprotective stimulus conditions. In the case of $K_{Na}1.2$, elevated Ca^{2+} would be predicted to inhibit the channel despite the raise in Na^+ [50,285]. The likely activators of these channels in IPC are upstream protein kinase signaling pathways (see above), while in the case of APC it is likely that volatile anesthetics are direct channel ligands.

While the use of volatile anesthetics is positively linked with reduced mortality in cardiac surgery [327], there is also evidence that repeated exposures can result in acute hepatitis [328]. Therefore, the identification of the channel that underlies the clinically important phenomenon of APC potentially paves the way for the development of novel $K_{Na}1.2$-targeted cardioprotective therapeutics [329]. However, an important caveat to these results is that we are the only laboratory that has to date investigated or provided any evidence for mitochondrial $K_{Na}1.2$. As such, validation of these findings by other laboratories will be necessary before moving toward any potential clinical applications.

Downstream mechanisms of protection due to mitochondrial K^+ channel opening

The mitochondrial PT pore is a fundamental arbiter of cell survival in IR injury (Figure 1) [71,330–334]. As such, numerous events at the mitochondrial level that are known to regulate the PT pore (e.g., mitochondrial Ca^{2+} overload, ROS generation, energetics, and pH) have been shown to interface with upstream signaling pathways implicated in cardioprotection (GSK-3B, $NO^{\cdot}$, signaling ROS, PKA, PKC, and others) [335–339]. However, despite a proposed central role for mitochondrial K^+ channels in IR protection (see Section 'Mitochondrial K^+ Homeostasis and Discovery of Mitochondrial K^+ Channels'), how such channels elicit downstream protective mitochondrial events is poorly understood.

There are numerous attractive hypotheses linking mitochondrial K^+ channels to the PT pore, and these can be roughly broken down into those dependent on membrane potential, and those that are not. Owing to the mitochondrial K^+ cycle (Figure 2), it is apparent that opening of a mitochondrial K^+ channel may serve (coupled with a KHE) to uncouple mitochondrial oxidative phosphorylation. Mild uncoupling of Ox-Phos alone is known to be cardioprotective [22,340–342] and may have many salutary effects on IR injury, such as those described in the following paragraphs.

ROS generation

Tissue reperfusion following ischemia is known to trigger a burst of ROS, and it is also known that mitochondrial ROS generation is exquisitely sensitive to membrane potential [77,78]. As such, it has been proposed that mild uncoupling may serve to depress ROS generation in early reperfusion [343–345]. This may be achievable via opening of a mitochondrial K^+ channel [244,245,346–348]. In addition, APC protection may also decrease ROS at reperfusion via mild uncoupling [349–351]. These findings would appear to position mitochondrial K^+ channel opening upstream of a decrease in pathologic ROS.

However, the interplay between ROS and mitochondrial K^+ channels in the setting of IR injury is much more complicated. Specifically, it is well known that low levels of ROS (termed 'signaling ROS') are in fact required for the cardioprotective effects of IPC [352–356] and APC [357]. This is consistent with the notion that ROS is hormetic, and indeed, low levels of ROS alone are known to confer IR protection [353,358,359]. In addition, the mitoK_{ATP} channel is redox-sensitive and opens in response to a variety of ROS [360]. These findings position mitochondrial K^+ channel opening downstream from signaling ROS.

Still further complication arises from the claim that opening of a mitochondrial K^+ channel itself can trigger ROS generation by complex I [361], which would position channel opening upstream of signaling ROS. Overall, it appears that the relationship between mitochondrial K^+ channels and ROS generation may be bi-directional, with signaling ROS and mitochondrial K^+ channel opening perhaps exhibiting an amplification loop behavior during the early trigger phase of cardioprotection, leading to an overall decrease in pathologic ROS at reperfusion. Unfortunately, beyond the brute-force application of antioxidants, the evidence for a role of ROS in transmitting a protective signal as part of an IPC or mitochondrial K^+ channel signaling cascade is

somewhat limited. Clearly, much remains to be done, in elucidating the order of events relating mitochondrial K^+ channels and ROS in IR protection. Recent progress in identifying the molecular constituents of mitochondrial K^+ channels (see Sections 'Mitochondrial K_{ATP} Channel: Composition, Pharmacology, Regulation, Role in IR Protection; Mitochondrial $K_{Ca}2.x$ and $K_{Ca}3.1$ Channels: Composition, Pharmacology, Regulation, Role in IR Protection; Mitochondrial $K_{Ca}1.1$ Channels and Mitochondrial $K_{Na}1.x$ Channels; and Mitochondrial $K_{Na}1.x$ Channels') may also permit the identification of redox-sensitive residues (e.g., cysteine and methionine) within these proteins that are responsible for the interplay of these channels with ROS.

Mitochondrial Ca^{2+} and autophagy

Another potential benefit of mild uncoupling via opening of a mitochondrial K^+ channel would be the prevention of mitochondrial Ca^{2+} uptake, which is driven by the membrane potential. As such, opening of a mitochondrial K^+ channel may prevent mitochondrial Ca^{2+} overload [362,363], thus serving to prevent PT pore opening. A third potential benefit of mild uncoupling downstream from mitochondrial K^+ channel opening could be the triggering of mitophagy [364], which is itself known to be cardioprotective [342,365]. The ability of mitochondrial K^+ channel opening to regulate mitophagy has not been rigorously investigated, although it was shown that the mitoK$_{ATP}$ opener DZX induces mitophagy in murine hearts [366], and it is also known that isoflurane induces cardiac mitophagy [367].

In considering the above phenomena, a caveat should be rendered regarding any link between mitochondrial K^+ channel opening and mitochondrial uncoupling, in terms of the size of the K^+ conductance involved. Specifically, it is known that opening of the mitoK$_{ATP}$ channel only drops membrane potential by 1–2 mV in isolated cardiac mitochondria (from its baseline value of ∼180 mV) [368]. Thus, any such uncoupling mediated by a mitoK$_{ATP}$ channel is likely to be insufficient to affect mitochondrial function (i.e., ATP production), although it could be sufficient to affect the driving force for mitochondrial ion fluxes. In contrast, the larger conductance of mitochondrial BK channels renders them more attractive candidates for inducing uncoupling. In this regard, we have found that the $K_{Na}1.x$ opener biothionol (which is cardioprotective, [307]) is also capable of inducing mitochondrial uncoupling in cardiomyocytes (unpublished data). In addition, the $K_{Na}1.x$ opener niclosamide was recently reported to uncouple mitochondria [369]. It is not yet known if the uncoupling effect of niclosamide is mediated by a mitochondrial K^+ channel or is capable of conferring cardioprotection.

Membrane potential-independent effects and volume

Beyond effects that depend on membrane potential, mitochondrial K^+ channels are thought to play a role in regulating mitochondrial volume [74] (see Section 'Mitochondrial K^+ Homeostasis and Discovery of Mitochondrial K^+ Channels' and Figure 2). Thus, it is possible that mild swelling associated with mitochondrial K^+ channel opening [370] may be part of a protective signaling cascade. Mitochondrial volume has been historically linked to respiratory function, with the transition between classical respiratory state 4 (quiescent) and state 3 (phosphorylating) being associated with a contraction of the mitochondrial matrix. [75,76,371]. As such, mild swelling would be expected to coincide with a lower overall mitochondrial respiratory function. How this would lead to protection against IR injury is not clear.

Alternatively, mitochondrial swelling could confer protection by many other mechanisms as follows: (i) by improving efficiency of the creatine kinase energy shuttle, for example, by changing the distance between inner and outer mitochondrial membranes. (ii) By regulating the supra-molecular assembly of respiratory chain complexes and super-complexes [372]. For example, it has been shown that mitochondria from hearts protected by APC had improved ATP synthase function [335]. Furthermore, it has recently been proposed that the cardioprotective drug SS-31 (Bendavia) [373] may confer protection via the stabilization of super-complexes involving cardiolipin [374–376]. (iii) Mild swelling could interfere with PT pore assembly [332,377]. (iv) Mild swelling would also be expected to dilute the contents of the mitochondrial matrix, which may directly affect the activity of enzymes in the tricarboxylic acid cycle by lowering substrate concentrations, or may affect concentrations of important mitochondrial enzyme allosteric regulators such as Ca^{2+}, NADH, acetyl-CoA, and phosphate. (v) By physiologic coupling to other mitochondrial channels or transporters that can sense volume or osmolarity.

From a perspective of long-term protective benefits of mitochondrial K_{ATP} opening, it has been shown that the treatment of cells with DZX causes mild *in situ* mitochondrial swelling, which can trigger a signaling cascade involving cyclic AMP responsive binding element (CREB) and NF-κB, leading to resistance to apoptosis [378]. Thus, there are clearly cell signaling mechanisms triggered by mitochondrial volume changes, which may play an important role in IR protection and remain to be determined.

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In summary, the events linking mitochondrial K^+ channel opening to protection from IR injury are currently poorly understood, both at the mechanistic and molecular levels. It is hoped that the future availability of specific mitochondrial K^+ channel ligands (facilitated by the molecular identification of these channels) will permit the independent interrogation of mitochondrial K^+ channel opening and swelling as a signaling trigger mechanism, to elucidate these downstream pathways.

Physiologic role of mitochondrial K^+ channels beyond cardioprotection

Given the importance of K^+ as a cytosolic solute and the conserved nature of mitochondrial K^+ channels, it is important to consider the endogenous physiologic role(s) of these channels in the cell, beyond protection from IR injury. Such considerations could also provide insights into novel mechanisms of regulating mitochondrial function.

At the organism level, as already discussed in Section 'Mitochondrial $K_{Ca}2.x$ and $K_{Ca}3.1$ Channels: Composition, Pharmacology, Regulation, Role in IR Protection', mammalian K_{ATP} channels play important roles in glucose-stimulated insulin secretion in pancreatic β-cells [379], and these channels are the pharmacologic target of the widely used antidiabetic sulfonylurea class of drugs. In addition, mammalian BK channels are broadly recognized to play roles in regulating vascular smooth muscle tone [380,381], in muscle relaxation [382], in regulating circadian rhythms [383,384], and in the function of neurons in the dorsal root ganglion [89]. Both $K_{Na}1.x$ paralogs are highly expressed in the brain, and the majority of research on endogenous $K_{Na}1.x$ channels has been conducted in neurons, focused mainly on $K_{Na}1.1$ [287]. Whether any of these functions attributed to $K_{Na}1.x$ channels are in fact due to such channels located in mitochondria is not known.

Evidence for a direct physiologic function of mitochondrially localized K^+ channels beyond their role on protection against IR injury is very sparse. Historically, uncoupling has been viewed as an important contributor to basal metabolic rate, perhaps best envisioned in brown adipose tissue (BAT), which burns fat to generate heat via mitochondrial uncoupling. The discovery of homologs of the BAT uncoupling protein (now called UCP1, [385]) in other tissues has led to a consensus that these proteins (UCP2–5, [386–389]) may serve a role in regulating whole-organism energy expenditure [390–392]. An alternative uncoupling mechanism has also recently been proposed, involving a nonspecific pore formed by the c-subunit of the ATP synthase in mitochondria [393]. Whether uncoupling by opening of a mitochondrial K^+ channel (presumably of the $K_{Na}1.x$ variety — see Section 'Downstream Mechanisms of Protection by Mitochondrial K^+ Channel Opening') is capable of having a similar effect on whole-organism energy balance remains to be seen, although it is exciting that the $K_{Na}1.x$ activator niclosamide is reported to have an antiobesity effect similar to the uncoupler 2,4-dinitrophenol [369]. This result potentially positions mitochondrial $K_{Na}1.2$ as a candidate antiobesity drug target.

Outlook

In the roughly three decades, since the discovery of IPC [394] and APC [395], there has been a plethora of research devoted to understanding the molecular underpinnings of these phenomena. Although mitochondrial K^+ channels were identified as candidate players early in this research arc, only in the past 4 years have viable molecular identities been assigned to these channels: $K_{Na}1.2$ [307], $K_{Ca}2.2/K_{Ca}2.3$ [180], and KIR1.1 (with caveats as outlined in Section 'Mitochondrial K_{ATP} Channel: Composition, Pharmacology, Regulation, Role in IR Protection') [95]. These identities can now be used to develop novel molecules to afford protection of organs such as the heart and brain from ischemic injury in a clinical setting.

Finally, it is noteworthy that the field of mitochondrial K^+ channel research has used model organisms at multiple stages, including genetically engineered mice, *C. elegans*, plants [138], amoeba [139], and trypanosomes. In addition, the field exists as a clear demonstration of the importance of basic biomedical research toward understanding a clinically relevant phenomenon in humans. The discoveries made regarding mitochondrial K^+ channels in the past 4 years provide a rich resource for future development of clinical therapies.

Abbreviations

5-HD, 5-hydroxydecanoate; APC, anesthetic preconditioning; BAT, brown adipose tissue; BK, big conductance of potassium; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; ChTx, charybdotoxin; DZX, diazoxide; EET, eicosaepoxytrienoic acid; IbTx, iberotoxin; IK, intermediate conductance; IPC, ischemic preconditioning; IR, ischemia–reperfusion; IUPHAR, International Union of Basic and Clinical Pharmacology; $K_{Ca}1.1$, channel

encoded by *Kcnma1*, also known as SLO1; $K_{Ca}2.1$, channel encoded by *Kcnn1*, also known as SK1; $K_{Ca}2.2$, channel encoded by *Kcnn2*, also known as SK2; $K_{Ca}2.3$, channel encoded by *Kcnn3*, also known as SK3; $K_{Ca}3.1$, channel encoded by *Kcnn4*, also known as IK, SK4; $K_{Ca}5.1$, channel encoded by *Kcnu*, also known as SLO3; KHE, K^+/H^+ exchanger; KIR, inwardly rectifying potassium channel; $K_{Na}1.1$, channel encoded by *Kcnt1* (formerly *Slo2.2*), also known as Slack, $K_{Ca}4.1$, SLO2.2; $K_{Na}1.2$, channel encoded by *Kcnt2* (formerly *Slo2.1*), also known as Slick, $K_{Ca}4.2$, SLO2.1; mitoK_{ATP}, ATP-sensitive mitochondrial potassium channel; NCX, sodium/calcium exchanger; NHE, sodium/proton exchanger; $NO^{\cdot}$, nitric oxide; PKA, cAMP-dependent protein kinase; PKC, Ca^{2+}/diacylglycerol-dependent protein kinase; PKG, cGMP-dependent protein kinase; PT, permeability transition; RCK, regulation of conductance of K^+; ROMK, renal outer medullary potassium channel; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum calcium-ATPase; SK, small conductance; SLO, slowpoke; *Slo2*, *C. elegans* gene encoding the single isotype SLO2 K_{Ca} channel; SloTx, slotoxin; SUR, sulfonylurea receptor.

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Competing Interests
The Authors declare that there are no competing interests associated with the manuscript.

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