

# Physiology of potassium channels in the inner membrane of mitochondria

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**Abstract** The inner membrane of the ATP-producing organelles of endosymbiotic origin, mitochondria, has long been considered to be poorly permeable to cations and anions, since the strict control of inner mitochondrial membrane permeability is crucial for efficient ATP synthesis. Over the past 30 years, however, it has become clear that various ion channels—along with antiporters and uniporters—are present in the mitochondrial inner membrane, although at rather low abundance. These channels are important for energy supply, and some are a decisive factor in determining whether a cell lives or dies. Their electrophysiological and pharmacological characterisations have contributed importantly to the ongoing elucidation of their pathophysiological roles. This review gives an overview of recent advances in our understanding of the functions of the mitochondrial potassium channels identified so far. Open issues concerning the possible molecular entities giving rise to the observed activities and channel protein targeting to mitochondria are also discussed.

**Keywords** Mitochondria · Potassium channel · Potassium transport · Ischaemia · Apoptosis · ROS 17/2.8

## Introduction

Mitochondria consist of an outer membrane, generally considered to be freely permeable to metabolites and ions, an inner membrane (IMM), where oxidative phosphorylation takes place, an intermembrane (periplasmic) space, and the matrix, enclosed by the IMM, where important metabolic processes such as the citric acid cycle and fatty acid beta-oxidation occur. The electrochemical driving forces for ion movement across the IMM are of considerable magnitude, given the very high negative membrane potential created by the respiration-driven efflux of protons from the matrix during oxidative phosphorylation. The electrochemical gradient has two components: (1) the voltage difference—negative inside—across the lipid membrane ( $\Delta\Psi_m$ ); (2) a chemical component, i.e. a different concentration of ions on the two sides of the membrane ( $\Delta p_{\text{ion}}$ ). The combination of these two factors determines the thermodynamically favourable direction for ion movement across a membrane. In energised mitochondria, its value for protons corresponds to  $-180/-200$  mV, (negative on matrix side), with  $\Delta\Psi_m$  as the major component. The activity of  $K^+$  in the mitochondrial matrix is often taken to be close to that of the cytoplasm, since early studies produced estimates of 83–160 nmol/mg protein, and matrix volume is in the order of 1  $\mu\text{l}$ /mg protein [131], but the matter is debated. Using a fluorescent indicator and permeabilised cells, Zoetewij and colleagues [199] estimated a mitochondrial  $K^+$  concentration of only 15 mM. Kaasik and co-workers have found that depolarisation of mitochondria in situ is

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accompanied by swelling (itself a controversial point [98]), which they have attributed to  $K^+$  influx [98, 157]. The explanation offered by the authors is the inhibition of the  $K^+/H^+$  exchange activity as the transmembrane potential decreases [98]. This would imply that  $[K^+]_{\text{matrix}} < [K^+]_{\text{cytosol}}$ . The alternative or concurrent explanation ought to be considered that the voltage drop may be associated with activation of  $K^+$  channels in the IMM (mitoK<sup>+</sup>), increasing the  $K^+$  conductance and thus influx despite the decrease of the driving force (“negative resistance”). The  $\Delta\mu K^+$  may thus be as low as about 150 mV if  $[K^+]_{\text{matrix}} = [K^+]_{\text{cytosol}}$  or as high as 210 mV if  $[K^+]_{\text{matrix}}$  is tenfold lower than  $[K^+]_{\text{cytosol}}$ , but in either case, there is no question that the direction of an electrophoretic  $K^+$  flux in even partially energised mitochondria is inward. Given the high driving forces for ion flux, channels would be expected to be under tight control and indeed can be found in extremely low abundance and/or to be open for very short times to maintain the low permeability to ions required to exploit the proton-motive force for ATP generation. Mitochondrial inner membrane ion channels selective for  $Ca^{2+}$ ,  $K^+$ , or anions have been functionally and pharmacologically characterised using both the methods of classical bioenergetics and electrophysiology (e.g. [17, 18, 34, 47, 137, 138, 181, 200]). Information about the behaviour of these channels has accumulated by now also at the single channel level, in isolated mitochondria but also in intact cells and in some cases at whole-organ level.

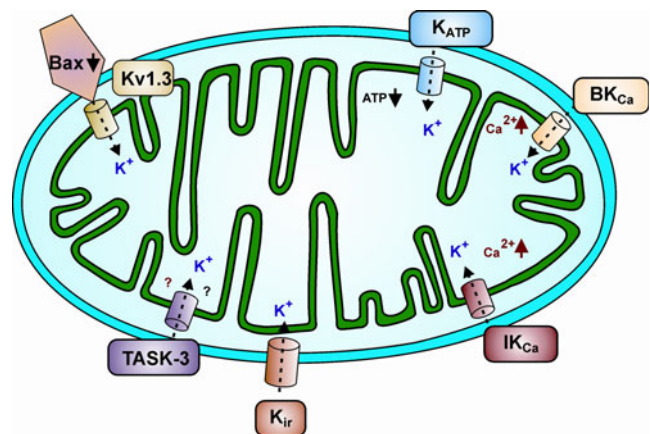
Potassium fluxes (the “ $K^+$  cycle”) in suspensions of isolated mitochondria have been monitored via swelling assays (based on the observation that net uptake of  $K^+$  salts is accompanied by osmotically obligated water influx resulting in matrix swelling [63]), measurements of changes in mitochondrial redox potential, respiration and  $\Delta\Psi_m$  and by direct determination of  $K^+$  transport using potassium-selective electrodes (for excellent reviews on  $K^+$  transport see, e.g. 19, 24, 62, 66, 134]. Furthermore, pathways allowing potassium flux through the IMM can be revealed directly by patch clamp recordings from mitoplasts, i.e. swollen mitochondria with only remnants of the outer membrane left, and thus suitable for electrophysiological experiments [200]. Electrophysiological planar lipid bilayer recordings of channels, originally in proteoliposomes containing purified mitochondrial membrane proteins or in isolated membrane vesicles, also proved very useful [18, 181, 183]. In vitro reconstitution of mitochondrial ion channels using droplet interface bilayer is emerging as a convenient technique, given that direct patch clamping of mitoplasts is technically rather difficult [114].

The pharmacological characterisation of the mitochondrial potassium channels has allowed the exploitation of the identified inhibitors and activators to elucidate the

physiological roles of these channels in the organism. This review focuses primarily on this latter aspect, given that excellent recent reviews summarising the electrophysiological properties of mitochondrial  $K^+$  channels are available [18, 137, 181, 183, 200]. The channels that have been identified so far are discussed below and are shown in Fig. 1.

### The mitochondrial potassium cycle

Using the methods of bioenergetics mentioned above, it was established that in isolated mitochondria, the high negative electrical membrane potential drives an electrophoretic  $K^+$  influx [66].  $K^+$  is the major monovalent cation in the cytoplasm and is consequently an important ion mediating changes in volume when the  $K^+$  conductance of the inner membrane is altered. Net uptake of  $K^+$  salts is accompanied by water, permeating through mitochondrial aquaporins [4, 31]. Excess matrix  $K^+$  is then exported by the electroneutral  $K^+/H^+$  antiporter, which is regulated to sense volume changes via changes in pH and  $[Mg^{2+}]$  and uses the energy stored in the proton gradient to export  $K^+$  from the matrix. A continuous electrophoretic influx of cations followed by their release via an electroneutral mechanism would, however, result in a futile cycle dissipating the membrane potential. Respiring mitochondria in  $K^+$ -containing media undergo massive swelling upon addition of the potassium ionophore valinomycin (which allows the rapid electrophoretic uptake of  $K^+$ ).



**Fig. 1** Mitochondrial potassium channels. The figure summarises the potassium channels identified by far in the outer and inner mitochondrial membranes. In the outer membrane, an inward rectifying channel ( $K_{Ir}$ ) has been characterised, while in the inner membrane activities ascribed to an ATP-dependent potassium channel ( $K_{ATP}$ ), to big and intermediate conductance calcium-activated potassium channels ( $BK_{Ca}$  and  $IK_{Ca}$ , respectively) and to Kv1.3 voltage-gated potassium channels have been recorded by electrophysiological techniques and some were also detected by electron microscopy. Furthermore, immunohistochemistry located TASK-3 two-pore potassium channel to the IMM. See text for details and references

Simultaneous addition of nigericin (which promotes the electroneutral exchange of  $H^+$  and  $K^+$ ) does prevent swelling but causes permanent depolarisation and “uncoupling” (i.e. electron transport through components of the respiratory chain is not anymore coupled to ATP production). In intact mitochondria, energy dissipation is limited by the fact that channels and electrophoretic uptake pathways (uniports) are strictly regulated. The so-called  $K^+$  cycle thus can potentially modulate the tightness of coupling between respiration and ATP synthesis, thereby maintaining a balance between energy supply and demand in the cell and controlling the magnitude of the  $\Delta\Psi_m$  and  $\Delta\tilde{\mu}_H$ . These latter parameters are also relevant for reactive oxygen species (ROS) production by mitochondria (see below), mitochondrial dynamics and mitophagy [165, 189]. The  $K^+$  cycle also ensures mitochondrial volume regulation, preventing excessive matrix swelling, thus maintaining the structural integrity of the organelle, or preventing contraction, which would inhibit respiration [86]. Volume changes of mitochondria may a priori have an impact also on calcium signalling within the cells by altering the architecture of contact points between the endoplasmic reticulum and mitochondria [60, 152, 176]; however, this hypothesis is still to be tested. In favour of this idea, it has been proposed that valinomycin-induced increased IMM potassium conductance in brain mitochondria may provide resistance to pathological calcium challenges via matrix volume changes [81]. Besides opening of the so-called permeability transition pore [201],  $K^+$  influx may lead to formation of the so-called donut-shaped, toroidal mitochondria during hypoxia and reoxygenation [115]. The significance of such change in the mitochondrial shape, however, is still undefined. Interestingly, a recent report suggests a crucial role for mitochondria in rapidly sequestering extracellular  $K^+$  that is taken up across the plasma membrane in astrocytes [108]. Increases in extracellular potassium concentration ( $[K^+]_o$ ) can occur during neuronal activity and under pathological conditions such as ischaemia, leading to a compromised neuronal function. Astrocytes contribute to the clearance of excess  $K^+_o$ , apparently by taking up potassium from the cytoplasm into mitochondria. Thus, mitochondria seem to act not only as a safety sink for cytoplasmic calcium but also for excess extracellular potassium.

Finally, the  $K^+$  cycle seems to regulate mitochondrial ROS production, although this process is not yet completely understood. The major (although not the only) sites of superoxide (ROS) formation in mitochondria are recognised to be complexes I and III of the respiratory chain [121, 168]. The regulation of ROS production is different in the two cases, and this may account in part for a certain degree of confusion surrounding the mechanism of preconditioning by

$K^+$  channel activation (see below). At complex I  $O_2^-$  generation seems to correlate largely with the reduction state of the matrix-facing flavine mononucleotide, which is in turn linked to that of the NAD/NADH couple. The latter is controlled by the relative rates of supply and disposal (by the downstream components of the respiratory chain) of reducing equivalents. Hence, an increased supply (e.g. by reverse electron flow from complex II) or a diminished disposal (e.g. due to an increase of  $\Delta\tilde{\mu}_H$ , the thermodynamic “force” opposing proton pumping and therefore the associated electron transfer) will increase ROS production. Depolarisation is expected to reduce ROS production at this site, and in fact, ROS production by succinate- or pyruvate/malate-oxidising mitochondria has been observed to be reduced by uncouplers [168]. Thus, opening of mito $K^+$  channels would be expected to reduce ROS production if complex I is the major site of production, as may well be the case when oxygen is reintroduced after a period of anoxia. At complex III, superoxide production is understood to be due to one-electron reduction of oxygen by ubisemiquinone, which is greatly enhanced by the presence of the inhibitor antimycin A. Ubisemiquinone at complex III is also likely [28, 29, 112, 188] to be the source of superoxide produced upon depolarisation of in situ (Jurkat cells) mitochondria with classical uncouplers such as carbonyl cyanide-p-trifluoromethoxyphenylhydrazone or 2,4-dinitrophenol, which we have observed (Sassi et al. unpublished data). The mechanism leading to an increased ROS production in this case is more obscure, but it is considered to be related to an increase in the occupancy of the semiubiquinone radical at the “o” centre of the bc<sub>1</sub> complex [120, 122]. Interestingly, recent quantitative modelling studies have indicated that complex III, and actually the whole respiratory chain, can exhibit “bistability”, i.e. function either in a high- or a low-ROS production steady state, correlating with the concentration of electron donor species at the appropriate sites. Which state is entered would depend on initial conditions or externally applied transient perturbations such as hypoxia [168, 169]. It is clear at any rate that the effect of alterations of  $\Delta\tilde{\mu}_H$  on ROS production depends on the characteristics of the particular system under study (see also [25]. In keeping with this complicated and variable setting, some experimental results suggest that depolarisation of the IMM and increase of the respiratory rate induced by opening of a  $K^+$  channel may increase the “leak” of electrons from the respiratory chain to form superoxide anion, i.e. ROS (e.g. [6, 35, 86, 109, 135]), while others indicate that the same effect is obtained by inhibition ([178], Leanza et al. unpublished). In other studies, activation of mitochondrial potassium channels has instead been reported to reduce/prevent ROS production (e.g. [52, 53, 56, 85, 110]).

Evidence has been accumulating of important roles of mitochondrial potassium channels in cytoprotection on one hand (mitoK<sub>ATP</sub> and mitoBK<sub>Ca</sub>) and in apoptosis on the other (Kv1.3 and mitoBK<sub>Ca</sub>). We review below the current information about the channels and their contribution to protection from ischemic damage and to the regulation of cell death. Due to their involvement in these processes, mitoK<sup>+</sup> channels are considered now promising therapeutic targets for various pathologies, and a possible role in metabolic diseases can also be envisioned [34].

### The mitochondrial ATP-dependent potassium channel

An ATP-sensitive potassium channel has been described in mitochondria of various tissues in mammals as well as in unicellular organisms and in plant mitochondria and has been proposed to correspond to the well-studied potassium “uniport” [9, 11, 66, 136, 182, 200]. The channel is selective for K<sup>+</sup> over Na<sup>+</sup>, and in the presence of Mg<sup>2+</sup>, it is inhibited by ATP on the cytoplasmic side of the IMM [91]. The Mg<sup>2+</sup>/ATP-inhibited channel can be reactivated by the plasma membrane ATP-sensitive K<sup>+</sup> channel (K<sub>ATP</sub> channel) openers cromakalim and diazoxide. The latter is much more effective on the mitochondrial than on the plasma membrane channel and is widely used to study the pathophysiological roles of mitoK<sub>ATP</sub> [49, 67, 116, 182]. Levosimendan is also considered a specific opener of mitoK<sub>ATP</sub> [104]. Obviously, differential sensitivity to pharmacological agents is a strong indication of structural differences between mitochondrial and plasma membrane K<sub>ATP</sub> channels. Other inducers of mitoK<sub>ATP</sub> activity include ROS [58, 71], GTP and GDP [129, 145], UDP [128], alkaline pH [20] and compound A [32], while NO [162], quinine [21], 5-hydroxydecanoic acid (5-HD) [94], glybenclamide [91] and long-chain acylCoA's [145] in addition to ATP and ADP, inhibit mitoK<sub>ATP</sub>.

The detailed pharmacological characterisation by classical bioenergetics and electrophysiology, initially performed mainly on rat liver mitochondria, later allowed determination of the presence of mitoK<sub>ATP</sub> in various organisms. In the non-photosynthesising free-living amoeboid protist *Acanthamoeba castellanii*, the electrophysiological (single-channel properties of the channel reconstituted into planar lipid membrane) and pharmacological (effect of specific modulators on bioenergetics of isolated mitochondria) profiles of the observed mitoK<sub>ATP</sub> channel display similarities to mammalian plasma membrane K<sub>ATP</sub> channel [103]. Swelling experiments have been used to assess the effects of some of the above-mentioned substances, known to modulate the mammalian mitoK<sub>ATP</sub> channel, on mitochondria of protozoan parasites trypanosomatids *Trypanosoma cruzi* (which causes Chagas' disease) and

*Crithidia fasciculata* [42]. MitoK<sub>ATP</sub> activity was identified also in mitochondria of the nematode worm *Caenorhabditis elegans* [194]. Furthermore, in plant mitochondria, which possess a very active K<sup>+</sup> cycle, a K<sup>+</sup> uniport pathway with characteristics of mitoK<sub>ATP</sub> has been observed to sustain an ion flow sufficient to uncouple respiration [38, 95, 141, 142]. Very recently, the first patch clamp experiments on plant mitochondria identified mitoK<sub>ATP</sub> also at the level of single channel activity [45]. At the same time, a channel with similar single channel and pharmacological properties was observed in bilayer experiments using plant mitochondria inner membrane vesicles [124]. In mammalian mitochondria, mitoK<sub>ATP</sub> channel activity, revealed by classical bioenergetic techniques (see above), or electrophysiological techniques patch clamp [44, 91] and planar bilayer (e.g. [20, 39, 128, 129]) was observed in liver, heart, brain, kidney, skeletal muscle and human T lymphocytes. A recent study took advantage of the observation that plasma membrane K<sub>ATP</sub> is permeable to the heavy metal thallium [88] and developed a novel, Tl<sup>+</sup> fluorescence-based assay to measure mitoK<sub>ATP</sub> channel activity [195].

Concerning the molecular identity of mitoK<sub>ATP</sub>, a definitive identification is not available yet. As for its plasma membrane-located counterpart (K<sub>ATP</sub> channel), inward rectifying potassium channel subunits (Kir6.1 or Kir6.2) together with the regulatory subunit sulphonylurea receptor (SUR) have been proposed to give rise to mitoK<sub>ATP</sub> activity. Neither KIR6 nor SUR genes contain mitochondrial target sequences, and KIR6/SUR proteins are not found in mitochondrial proteome databases or prediction engines [75, 140]. A recent study using specific antibodies and SUR knockout mice identified short-form splice variants of SUR2 in mitochondria [197]. Mice with the SUR2 gene deleted displayed, however, an enhanced cardioprotection, contrary to what may have been expected (see below), which was associated with a protected mitochondrial phenotype resulting from enhanced K<sup>+</sup> conductance that partially dissipated  $\Delta\Psi_m$  [1]. Targeted expression of Kir6.2 in mitochondria protected against hypoxic stress [118] and localisation of Kir6.2-GFP to mitochondria in COS-7 cells was promoted by protein kinase C [61]. Kir6.1 has instead been located prevalently to the endoplasmic reticulum when expressed in a heterologous system as Kir6.1-GFP and was shown to play a role in modifying Ca<sup>2+</sup> release from intracellular stores [133]. Over-expression of proteins in heterologous systems might however lead to mistargeting; therefore, proving localisation using some alternative method is generally recommended. Unfortunately, mitochondria from Kir6.2 knockout animals [126] have not been studied so far concerning the mitoK<sub>ATP</sub> activity at single channel level to clarify this question. An alternative

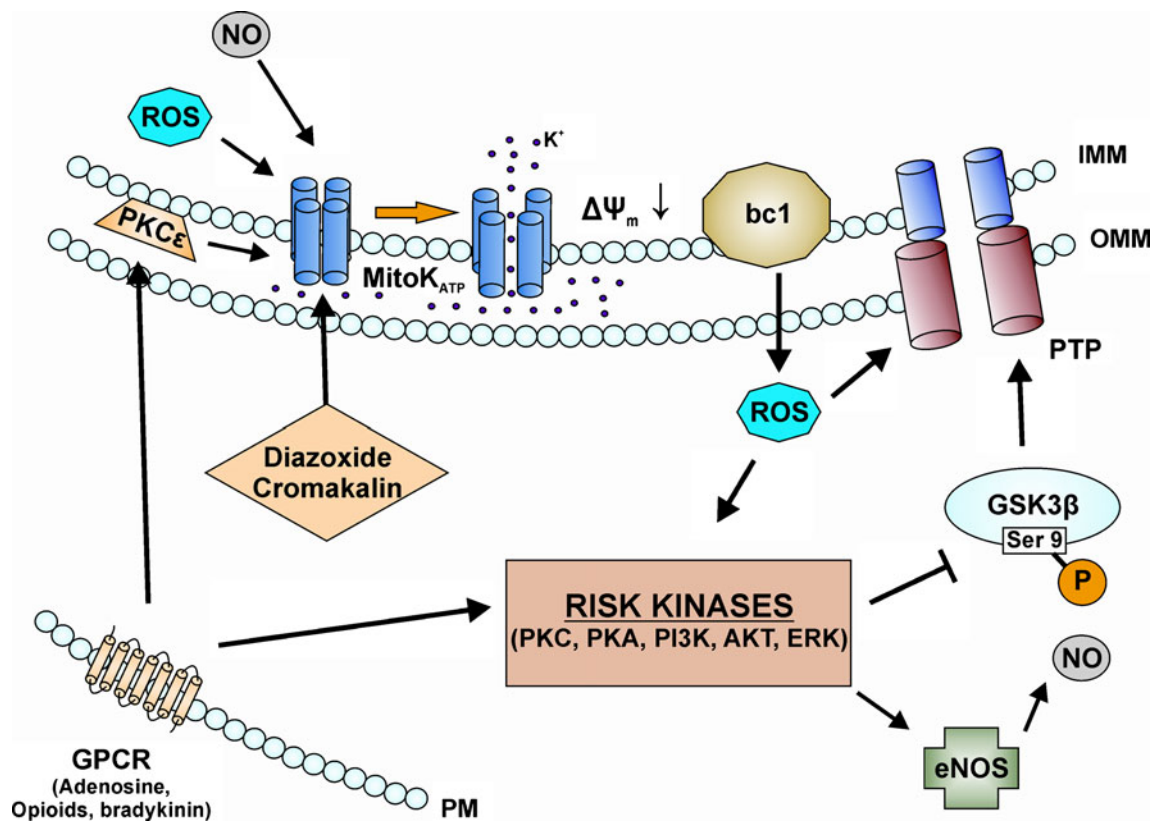


hypothesis on the molecular nature of this channel envisions a supercomplex comprising succinate dehydrogenase, complex V (ATP-synthase), the adenine nucleotide translocator (ANT), the phosphate carrier (piC) and ATP-binding cassette protein 1 (ABC1) [10]. Pharmacological evidence support the idea that complex II of the respiratory chain (succinate dehydrogenase) may be indeed a regulatory component of the mitoK<sub>ATP</sub> channel and ANT has been proposed to mediate the mitoK<sub>ATP</sub>-opener-induced potassium flux to the mitochondrial matrix [105]. Consistent with the idea of a supercomplex, the mitoK<sub>ATP</sub> channel inhibitor 5-HD allowed restoration of complex II and V activities and succinate oxidation in mice bearing a mutation in the mitofusin 2 gene (MFN2) [76], which encodes a mitochondrial outer membrane protein, crucial for mitochondrial fusion. The mechanism linking mutated MFN2 to mitoK<sub>ATP</sub> opening and OXPHOS defect remains to be elucidated and a direct interaction of MFN with mitoK<sub>ATP</sub> cannot be excluded. Overall, these findings leave the identity of the K<sup>+</sup> channel-forming subunit of mitoK<sub>ATP</sub> unknown. Given that this channel activity is present in various organisms with sequenced genomes, a comparative genomics/proteomics approach, recently applied with success to identify the mitochondrial calcium uniporter [17, 47], may help the molecular identification of mitoK<sub>ATP</sub>.

MitoK<sub>ATP</sub> is involved in the regulation of mitochondrial ionic homeostasis as described above. Furthermore, importantly, its activation has been observed to provide cytoprotection against ischemic damage (e.g. [24, 41]). The reperfusion of ischemic myocardium induces a burst-like release of superoxide (from the electron transport chain), which is rapidly transformed into hydrogen peroxide and hydroxyl radicals [202]. Although the burst of ROS during reperfusion lasts only several minutes, systolic and diastolic function remain impaired for several hours, a phenomenon termed “stunning”. ROS-induced activation of mitoK<sub>ATP</sub> channels may provide endogenous protection from stunning [119]. ROS and Ca<sup>2+</sup> overload at reperfusion are thought to induce opening of the mitochondrial permeability transition pore (PTP; a large, a specific IMM channel opened by matrix Ca<sup>2+</sup> and oxidative stress [12, 201], with consequent cell death [13, 26, 79, 92, 139]). Ischemic preconditioning (IP), i.e. a series of brief sub-lethal ischemic periods, reduces necrotic damage by a subsequent massive ischaemia [84, 132]. Protection is also afforded by pharmacological preconditioning (and “post-conditioning”) with potassium channel openers [73], and at present, it is broadly (but not universally, see [78]) accepted that this is due at least in part to the activation of potassium channels localised in the mitochondria [9, 11, 48, 54, 65, 67, 130, 136, 146, 154, 160]. The links connecting activation of mitoK<sup>+</sup> channels, modulation of mitochondrial parameters, and

cytoprotection remain debated. Since in experiments with isolated mitochondria, an increase in mitochondrial volume antagonises the permeability transition, an analogous increase due to opening of mitoK<sup>+</sup> channels has been proposed to account for protection [81]. Increasing the permeability to K<sup>+</sup> of the IMM is expected to lower  $\Delta\Psi_m$  (see above; “mild uncoupling”), which in turn would decrease mitochondrial uptake of Ca<sup>2+</sup>, a key inducer of the PTP, and thus partly account for protection [52, 164]. In keeping with the ambiguity on the correlation between  $\Delta\Psi_m$  and ROS production (see above), this mild uncoupling has been found to reduce ROS production, thus again antagonising the onset of the PTP [52, 54, 56, 110], while other studies have observed instead a stimulation of ROS production [6, 35, 86, 109, 186]. Evidence has accumulated for a kinase signalling cascade initiated by these ROS, involving PKC $\epsilon$  and leading to inhibition of GSK3 $\beta$ , whose activity on mitochondrial targets is thought to facilitate the onset of the PTP ([96, 97, 117, 123, 159, 161, 174, 190, 198], reviews [40, 64, 130, 160, 192] (see Fig. 2)). As mentioned, the mitoK<sub>ATP</sub> channel has been shown to be redox-sensitive, so that it is itself modulated by specific reactive oxygen and nitrogen species and NADPH in a feedback loop possibly underlying its regulation in the context of ischemic preconditioning [150, 171].

In the majority of studies concerning cytoprotection, the selective mitoK<sub>ATP</sub> activator diazoxide has been used. This drug however has also channel-independent actions [80], namely it inhibits succinate dehydrogenase leading thus to depolarisation [89] and acts as a protonophore [89]. In addition, the effects of mitoK<sub>ATP</sub> channel openers on mitochondrial function may depend on the cell’s energetic state [51, 151], making the interpretation of the effect of mitoK<sub>ATP</sub> openers within the cells quite complex. A recent study showed that BMS-191095, a more specific opener of the mitochondrial ATP-regulated potassium channel [20, 72], which has been shown to provide cytoprotection in models of ischaemia reperfusion induced injury in various tissues, protected myoblasts from calcium ionophore A23187-induced injury, but not from H<sub>2</sub>O<sub>2</sub>-induced injury [121]. The authors concluded that BMS-191095-mediated cytoprotection observed in C2C12 myoblasts resulted probably from modulation of intracellular calcium transients, leading to prevention of calpain activation [121]. MitoK<sub>ATP</sub> has been implicated also in apoptosis and cell death in various systems. For example pre-treatment of cells with diazoxide exerts a protective effect against rotenone induced cell death [184] and against UV-induced skin damage [33]. It has also been reported to antagonise the division of leukemic cells by causing mitochondrial membrane depolarisation [90]. MitoK<sub>ATP</sub> is indeed emerging as new possible target for anticancer therapy [144].



**Fig. 2** Some of the signalling pathways possibly involved in mitoK<sub>ATP</sub>-mediated preconditioning. The cartoon represents a highly simplified view of a selection of the proposed models. Signals emanating from G-protein coupled receptors (GPCRs) (adenosine receptors in particular) during ischaemia lead to activation of PKC<sub>ε</sub> as well as classical PKC, PKA, PI3K, Akt and MAP kinases including ERKs (the “reperfusion injury salvage kinases” (RISK); [82, 83]. Some of these enzymes can also be activated by ROS. A pool of PKC<sub>ε</sub> associated with mitoK<sub>ATP</sub> [40, 93] can phosphorylate the channel and

induce its opening, which can also be caused by ROS, NO, and pharmacological agents. Activation of mitoK<sub>ATP</sub> results in depolarisation of the IMM, with consequent production of superoxide (represented as taking place at Complex III of the respiratory chain). ROS-activated RISK kinases phosphorylate GSK3β at Ser9, inhibiting it. GSK3β activity at mitochondria favours MPTP opening, and thus, its downregulation antagonises the permeability transition and cell death

In addition to their role in cytoprotection and regulation of cell death, mitoK<sub>ATP</sub> channels have been proposed to play a regulatory role in the control of energy metabolism and body weight [2]. Furthermore, mitoK<sub>ATP</sub> are a likely route for mitochondrial potassium uptake into astrocyte mitochondria, which also involves the mitochondrial population of connexin Cx43 [108]. Cx43 hemichannels have been located in the inner mitochondrial membrane in addition to sarcolemma and were shown to contribute to mitochondrial K<sup>+</sup> uptake [127]. Interestingly, mice lacking Cx43 are not protected against cellular injury upon pharmacological activation of mitoK<sub>ATP</sub> [87]. A study using patch clamping of the inner membrane of cardiac mitochondria found a reduced diazoxide-induced stimulation of mitoK<sub>ATP</sub> channels upon connexin inhibition by carbenoxolone or in Cx43-deficient mitochondria [155].

The role of mitoK<sub>ATP</sub> in physiological processes has also been studied in plants [38, 95, 124, 141, 142]. In that system, plant mitoK<sub>ATP</sub> (PmitoK<sub>ATP</sub>) channel is proposed to

be involved in the prevention of ROS formation and a “feedback” mechanism operating under hyperosmotic/oxidative stress conditions has been formulated: Stress conditions induce an increase in mitochondrial ROS production; ROS activate PmitoK<sub>ATP</sub>, which, in turn, dissipates the mitochondrial membrane potential, thus inhibiting further large-scale ROS production [142].

### Calcium-dependent potassium channels

Two types of calcium-activated potassium channels have been described in various cell types: mitoBK<sub>Ca</sub> (big conductance potassium channel) and mitoIK<sub>Ca</sub> (intermediate conductance channel). MitoBK<sub>Ca</sub> (KCa1.1) (reviews [136–138, 181, 200]), displaying a conductance of 100–300 pS, has been observed by direct patch clamping of mitoplasts of mammalian cells [170, 196] as well as in planar lipid bilayer experiments [173]. The channel has been identified

in a glioma cell line as well as in ventricular cells, rat skeletal muscle and brain [172]. Evidence for its presence in mitochondria has also been provided by Western blot, electron microscopy and immunofluorescence microscopy [172]. The channel is activated by micromolar concentrations of calcium and by the drugs 12,14-dichlorodehydroabietic acid (diCl-DHAA) [159], NS1619 and NS11021 [8, 23], CGS7181 and CGS7184 [113]. It is blocked by specific inhibitors charybdotoxin [74, 172], iberiotoxin [36, 37] and paxilline [85, 86]. One of the most often used drugs, NS1619, however has recently been proposed to induce matrix  $K^+$  and  $H^+$  influx through a nonspecific transport mechanism, independently of mitoBK<sub>Ca</sub> [3]. The mitoBK<sub>Ca</sub> channel  $\beta 4$  subunit, which acts as a regulatory component, was found in mitochondria, e.g. in brain [50, 149]. Interestingly, a charybdotoxin-insensitive BK channel activity has recently been described in brain [55], and the altered pharmacological behaviour of the mitoBK<sub>Ca</sub> channels was tentatively ascribed to regulation by the  $\beta 4$  subunit.

Concerning the molecular nature of mitoBK<sub>Ca</sub>, it seems to coincide with that of the PM-located BK<sub>Ca</sub> and/or its alternatively spliced forms. Interestingly, approximately 20% of the proteins (identified by proteomics) that interact with BK<sub>Ca</sub> are mitochondria-related [101]. Xu et al. [196] has reported the presence of a 55-kDa protein, recognised by an anti-BK<sub>Ca</sub> antibody in cardiac mitochondria, although the molecular weight of the full-length protein is 125 kDa. The BK<sub>Ca</sub>-DEC splice variant, when expressed in a heterologous system, was identified as a mitochondrial candidate [101]. In another study, insertless BK<sub>Ca</sub> was localised to the plasma membrane when expressed in Chinese Hamster Ovary cells, but a splice variant, different from BK<sub>Ca</sub>-DEC, was clearly targeted to mitochondria [187].

MitoBK<sub>Ca</sub> has been proposed to be activated under pathophysiological conditions that increase mitochondrial  $Ca^{2+}$  uptake, thus preventing excessive mitochondrial  $Ca^{2+}$  accumulation (by partially depolarising the IMM, the driving force for calcium influx is reduced) and may also play a physiological role to fine-tune mitochondrial volume and/or  $Ca^{2+}$  accumulation under conditions of, e.g. increased cardiac workload. Opening of mitoBK<sub>Ca</sub>, similarly to mitoK<sub>ATP</sub>, has been reported to protect against damage to the heart and other organs caused by ischaemia and reperfusion. Mitochondrial BK<sub>Ca</sub> channels, activated by hypoxia [36], seem to protect also cardiomyocytes isolated from chronically hypoxic rats, characterised by protection of the heart against injury caused by acute oxygen deprivation [27]. The protective effect of BK<sub>Ca</sub> openers has been attributed to increased matrix  $K^+$  uptake and volume, improved respiratory control [8], inhibition of mitochondrial  $Ca^{2+}$  overload [99, 158, 193] and prevention of permeability

transition pore (PTP) opening [36]. The effect of BK<sub>Ca</sub> modulators on death or survival is still ill-defined. BK<sub>Ca</sub> channel inhibition by pro-apoptotic Bax molecules, as observed in patch clamp experiments using recombinant Bax, might contribute to opening of the PTP, which takes place during cell death [37]. Opening of BK<sub>Ca</sub> in isolated brain mitochondria was shown to inhibit ROS production by respiratory chain complex I and was hypothesised to be beneficial for neuronal survival [110]. Although a BK<sub>Ca</sub>-less knockout mice is available [125], to our knowledge, no studies have yet addressed mitochondrial bioenergetics and electrophysiology in these mice.

Interestingly, mitoBK<sub>Ca</sub> activity has been revealed also in potato, tomato and maize mitochondria by swelling measurements [95], and a large-conductance  $Ca^{2+}$ -activated potassium channel has been described in potato tuber mitochondrial fractions incorporated into an artificial planar lipid membrane [106]. The mitoBK<sub>Ca</sub> channel in plant mitochondria may function as a possible signalling link between matrix calcium levels and the mitochondrial membrane potential.

MitoIK<sub>Ca</sub> (mitoKCa3.1; MIMIK) has been recently observed by patch clamping mitoplasts isolated from human colon carcinoma cells [46]. This channel has been detected in other cell types, namely in HeLa of human cervix adenocarcinoma origin and in mouse embryonic fibroblasts [163]. The channel is selectively inhibited by clotrimazole and TRAM-34. The biophysical and pharmacological properties of the mitoIK<sub>Ca</sub> and of the PM IK<sub>Ca</sub> channels of the same cells are indistinguishable, and the molecular weights of the IKs in the two membranes are the same [163]. MitoIK<sub>Ca</sub> may have a protective role similar to that proposed for mitoBK<sub>Ca</sub> and mitoK<sub>ATP</sub> channels; however, its physiological role has not been studied in detail so far. To check the role of mitoIK<sub>Ca</sub> in cell death, the membrane permeant TRAM-34 was used, but application of this inhibitor alone did not induce cell death. It will be interesting to study whether modulators of mitoIK<sub>Ca</sub> may influence cell survival when given together with other physiological stimuli.

### Mitochondrial Kv1.3 potassium channel

Kv1.3 is a highly selective channel expressed in the plasma membrane of various cells. It plays a crucial role in the regulation of proliferation in lymphocytes [30] as well as in other cell types. Its presence in the mitochondria (mitoKv1.3) has been reported in T lymphocytes [178], macrophages [191], hippocampal neurons [22] and presynaptic neurons [68], using patch clamp and/or immunocytochemistry. The molecular identification of the mitochondrial channel was obtained thanks to specific inhibitors margatoxin [22, 178]

and ShK [177] and to a genetic model consisting in Kv1.3-less CTLL-2 cytotoxic lymphocytes and CTLL-2 cells stably expressing Kv1.3 [178]. Interestingly, the channel described in the mitochondria of hippocampal neurons by patch clamp [22] displayed a different conductance with respect to the one found in lymphocytes, but both channels were sensitive to margatoxin. It should be emphasised that mitoKv1.3 activity has been found also in the mitochondria of genetically non-manipulated cells (Jurkat T lymphocyte, hippocampal neuron), indicating that in the case of CTLL-2/Kv1.3 cells, the channels' presence in the IMM is not due to an overexpression artifact and/or to mistargeting. Importantly, a hyperpolarisation of the mitochondrial membrane potential could be recorded upon inhibition of mitoKv1.3 with specific drugs, indicating that in energised mitochondria, the channel is normally active [178]. The question arises of how a voltage-gated outwardly rectifying channel might be active at the very negative mitochondrial membrane potential. MitoKv1.3 activity might be due to a decreased voltage dependence, possibly due to the absence of the regulatory  $\beta$  subunit [175] or to a marked hyperpolarising shift in the voltage dependence of activation ( $V_{1/2}$ ), possibly resulting from post-translational modifications, which however do not result in differences in migration on gels.

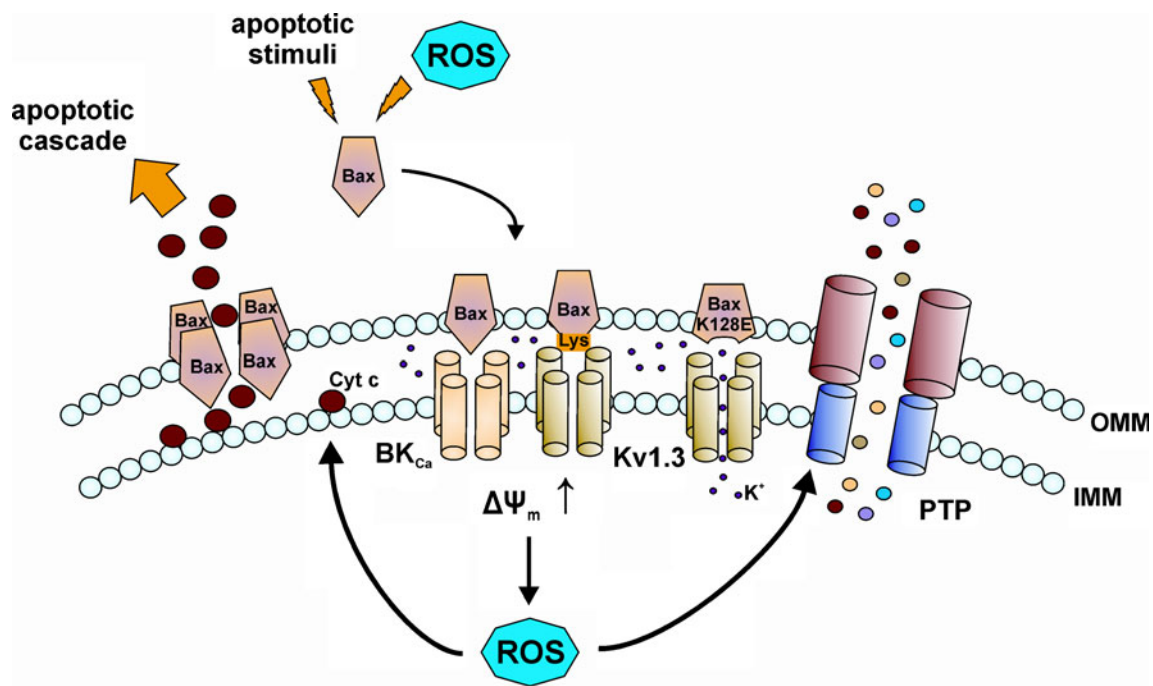
While PMKv1.3 activation is associated with cell proliferation [30], its mitochondrial counterpart has been found to play a role in cell death [77, 177, 179, 180]. Expression of mitochondria-targeted Kv1.3 was sufficient to sensitise apoptosis-resistant Kv1.3-less CTLL-2 T lymphocytes to a variety of death stimuli, while knockdown of Kv1.3 expression in human peripheral blood lymphocytes impaired apoptosis in these cells [177]. As to the mechanism, mitoKv1.3 was identified as a novel target of the pro-apoptotic Bcl-2 family protein, Bax, and a physical interaction between the two proteins was shown to occur, but only in apoptotic cells. Recombinant Bax inhibited Kv1.3 channel activity with an efficiency similar to that observed for the specific toxin inhibitors margatoxin or Shk. Incubating Kv1.3-positive isolated mitochondria with Bax or nanomolar concentrations of margatoxin or Shk-triggered apoptotic events, whereas mitoKv1.3-deficient mitochondria were resistant. The results indicated that during apoptosis, inhibition of IMM-located mitoKv1.3 by OMM-inserted Bax leads to hyperpolarisation, which results in the reduction of respiratory chain components and in enhanced production of ROS (see above) [177]. ROS is emerging as a key player in promoting cytochrome c release from mitochondria [102, 148]. Cytochrome c is normally bound in the intermembrane space to the IMM by association with cardiolipin [147]. Peroxidation of cardiolipin by ROS may lead to dissociation of cytochrome c, which becomes

released through the outer mitochondrial membrane into the cytosol by a still debated mechanism. Two well-supported models envision the formation of efflux pathways by oligomers comprising Bax and other proteins and/or activation of PTP and cristae remodelling [166, 167]. ROS are indeed able to oxidise thiol groups and thus to activate the PTP whose opening leads to depolarisation [43, 70, 201] and has been correlated to cytochrome c release (e.g. [111, 167]). Recombinant Bax and PM/mitoKv1.3-inhibiting toxins applied to isolated mitochondria induced an increase of ROS production followed by depolarisation and cytochrome c release. All these events, known to take place at mitochondria in various apoptotic models, were dependent on the presence of mitoKv1.3 and did not occur in mitochondria of Kv1.3-deficient CTLL-2/pJK cells. The signalling mechanism described above is summarised in Fig. 3. The block of mitoKv1.3 by Bax was found to depend on a particular, highly conserved residue in Bax. After insertion of activated Bax in the outer mitochondrial membrane, Bax lysine 128 is located in the intermembrane space [7] and mimics a crucial lysine in Kv1.3-blocking toxins. Mutation of Bax at K128 (BaxK128E) abrogated its effects on Kv1.3 and on isolated mitochondria. When expressed in apoptosis-resistant Bax/Bak-deficient mouse embryonic fibroblasts, BaxK128E was unable to reconstitute apoptosis induction by several stimuli [179]. Altogether, these results indicate that a toxin-like action of Bax on mitoKv1.3 is able to trigger at least some of the mitochondrial changes typical for apoptosis. Recent results from our laboratories suggest that the action of Bax can be replaced by specific membrane permeant inhibitors of Kv1.3 (Leanza et al., unpublished data). Whether mitoKv1.3 is the only channel of the Shaker voltage-gated potassium channel family located in mitochondria and whether its presence is obligatorily linked to its presence in the plasma membrane are still under investigation.

### Two-pore potassium channel TASK-3

Recently TASK-3 (TWIK-related acid-sensitive  $K^+$  channel-3; KCNK9), a two-pore potassium channel known to reside in the plasma membrane, was identified in mitochondria of melanoma and keratinocyte cells by immunochemical and molecular biology methods [156]. In the PM, TASK-3 has roles in apoptosis and tumorigenesis [143]. An investigation of the functional properties of the IMM TASK-3 channel by electrophysiology and/or spectroscopic methods remains to be performed. Notably, a TASK-3 knockdown melanoma cell line displays compromised mitochondrial function, suggesting that TASK-3 channels are functionally present in the mitochondria of the melanoma cells, and their function is essential for the survival of these cells [107].





**Fig. 3** Signalling pathways leading to cytochrome c release upon inhibition of mitoKv1.3 and mitoBK<sub>Ca</sub> by pro-apoptotic Bax. Apoptotic stimuli induce translocation of Bax from cytosol to the OMM. Bax is first inserted as a monomer exposing its lysine residue 128 to the inter-membrane space [7]. This residue is responsible for blocking Kv1.3 activity [179], leading to a hyperpolarisation, which in turn induces increase in ROS release [177]. ROS may detach

cytochrome c from the outer surface of the IMM and may lead to opening of PTP, with consequent membrane depolarisation. Release of cytochrome c from mitochondria may take place via oligomers of Bax or as a consequence of cristae remodelling due to the opening of the PTP. Inhibition of BK<sub>Ca</sub> by Bax has also been reported to activate PTP [37]. See text for further details

### Inward rectifier potassium channel of the outer mitochondrial membrane

Although the outer membrane of mitochondria is widely accepted to be freely permeable to small ions due to the presence of the voltage-dependent anion channel, VDAC (i.e. porin), a recent paper describes the discovery of an inwardly rectifying voltage-dependent potassium selective ion channel ( $K_{ir}$ ) in this membrane by patch clamp experiments [57]. The channel was found to be blocked by cesium, but was not affected by other classical general inhibitors TEA<sup>+</sup> and 4-aminopyridine. The activity was regulated by osmolarity and cAMP. The molecular nature of this channel as well as its single channel conductance remain to be clarified. It is to note that VDAC is able to adopt cation-selective conductance substates and also the completely closed, non-conductive state when incorporated into artificial bilayer [15, 16]. Thus, the channel identified by the group of Trotti might a priori represent a substate of VDAC, as the authors themselves discuss. In this respect, it is interesting to mention that classical porin activity in the OMM has indeed never been recorded, raising the question of whether the OMM is really freely permeable and whether a membrane

potential across it can exist. Furthermore, the outer membrane might contain contamination by ER via the so-called mitochondria-associated endoplasmatic reticulum membranes [69].

### Open questions

In summary, the mitochondrial potassium channels listed above are expressed in several different cell types and tissues, and the ones for which molecular candidates exist appear to represent subpopulations of channels present also in the plasma membrane. This raises questions concerning the mechanism of differential targeting of the channels and also casts some doubts on the effective presence of these channels in mitochondria, even though an analogous multiple localisation has been established for various other proteins (e.g. kinases). Several mitochondrial proteomic studies published so far have failed to recognise any mitochondrial channel except mitochondrial porin [185], BK<sub>Ca</sub> [101, 187] and  $\beta$  regulatory subunit of a non-identified voltage-gated potassium channel in rice [185]. This presumably reflects technical difficulties ascribable to the hydrophobic nature and especially to the low abundance

of channel proteins [59], rendering the identification of ion channels by mass spectrometry very difficult. Genetic approaches may be useful to obtain conclusive evidence on the molecular identity and localisation of some of the IMM channels. Using a biochemical approach, the observation that the abundance of a channel protein in various membranous fractions increases along with that of mitochondrial markers, while markers of contaminating membranes [PM and endoplasmic reticulum (ER)] decrease, may be considered good evidence for a mitochondrial location [77, 163]. Last but not least, observation of a channel by patch clamp in swollen mitoplasts, i.e. in the inner membrane, directly proves its presence in that membrane. A comparative proteomic/genetic approach combined with a systematic RNA interference screening may give the link between the genes coding for potassium channels and the mitochondrial potassium channels.

As to targeting, a classical, N-terminal mitochondrial sequence cannot be revealed in mitochondrial potassium channels (where identity is known), and the mechanism for their dual targeting is unknown. Examples of dual targeting are abundant [100]; for example, it has been reported that several members of the cytochrome P450 family (CYP) are targeted to both the ER and mitochondrial compartments [5, 153]. Dual targeting of CYP apoproteins to the ER and to mitochondria is modulated by an amino-terminal bipartite signal, which includes an ER targeting sequence followed by a cryptic mitochondrial targeting sequence. Activation of the cryptic signal is mediated through post-translational modification depending on cellular cAMP levels. By analogy, it can be hypothesised that effective targeting of potassium channels may depend on, e.g. cAMP levels. Targeting may also depend on other kind of post-translational modifications. Direct contact as well as biochemical interactions between ER and OMM membrane systems have been demonstrated [69], possibly giving rise to dual targeting. Furthermore, a recent paper indicates that even small changes in the length of the transmembrane domain of the viral potassium channel K<sub>esv</sub> alter its localisation between the plasma membrane and the mitochondria in mammalian cells [14]. Further work is required to clarify the mechanisms responsible for dual localisation of the IMM potassium channels.

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