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Novel channels of the inner mitochondrial membrane

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ABSTRACT

Along with a large number of carriers, exchangers and "pumps", the inner mitochondrial membrane contains ion-conducting channels which endow it with controlled permeability to small ions. Some have been shown to be the mitochondrial counterpart of channels present also in other cellular membranes. The manuscript summarizes the current state of knowledge on the major inner mitochondrial membrane channels, properties, identity and proposed functions. Considerable attention is currently being devoted to two K^+-selective channels, mtK_{ATP} and $mtBK_{Ca}$. Their activation in "preconditioning" is considered by many to underlie the protection of myocytes and other cells against subsequent ischemic damage. We have recently shown that in apoptotic lymphocytes inner membrane $mtK_V1.3$ interacts with the pro-apoptotic protein Bax after the latter has inserted into the outer mitochondrial membrane. Whether the just-discovered $mtIK_{Ca}$ has similar cellular role(s) remains to be seen. The Ca^{2+} "uniporter" has been characterized electrophysiologically, but still awaits a molecular identity. Chloride-selective channels are represented by the 107 pS channel, the first mitochondrial channel to be observed by patch-clamp, and by a ~400 pS pore we have recently been able to fully characterize in the inner membrane of mitochondria isolated from a colon tumour cell line. This we propose to represent a component of the Permeability Transition Pore. The available data exclude the previous tentative identification with porin, and indicate that it coincides instead with the still molecularly unidentified "maxi" chloride channel.

1. Introduction

Nature, or evolution, is thrifty. It's logical for it to utilize the same tools for as many tasks and in as many locations as possible. Especially if the tools in question are versatile and powerful signalling and transport appliances such as ion channels, transporters and pumps, meeting the needs of highly organized cells, full of membrane-bound compartments. Thus, the increasing evidence [1–3] that some proteins may be present in more than one cellular structure – often with very different abundance – should not be cause for surprise. The mechanisms involved are partly understood (for a discussion see [3]). The instances already known indicate that all subcellular compartments may share some proteins with others. Mitochondria have provided several examples [3–9]. In particular, the mitochondrial channels for which molecular candidates exist appear to represent subpopulations of channels present also elsewhere. The mechanism of dual targeting for these channels is not known. None of them harbours typical N-terminal mitochondrial targeting sequences. However, targeting information may comprise several distant amino acids spread throughout the entire protein, as in the case of mitochondrial carriers [10]. Alternatively, specific post-translational protein modifications and differential splicing have been hypothesized to account for organellar protein targeting [1].

Dual targeting represents a problem, in the sense that it is difficult to dispel the doubt that the presence in mitochondria might just be an artefact due to contaminations, insufficient selectivity of antibodies, etc. These doubts are reasonable, especially when dealing with

Abbreviations: ANT, Adenine Nucleotide Translocator; BHT, 3,5-di-t-Butyl-4-HydroxyToluene; BK_{Ca}, Big Ca-dependent K^+ channel; CaM, Calmodulin; CLIC, Chloride-selective Intracellular Channel; CSA, Cyclosporin A; diCl-DHAA, 12,14-dichlorodehydroabietic acid; $\Delta\psi_m$, mitochondrial transmembrane potential; DIDS, 4,4′-Dilsothiocyanatostilbene-2,2′-Disulfonic Acid; EM, Electron Microscopy; ER, Endoplasmic Reticulum; g, single-channel conductance; HCT, Human Colon Tumour; 5-HD, 5-HydroxyDecanoate; HP, Half-PTP; IK_{Ca}, intermediate Ca-dependent K^+ channel; IMAC, Inner Membrane Anion Channel; IMM, Inner Mitochondrial Membrane; IP, Ischemic Preconditioning; I/R, Ischemia/Reperfusion; K_{ATP}, ATP-sensitive K^+ channel; MAM, Mitochondria-Associated Membranes; MEF, Mouse Embryonic Fibroblast; MgTx, Margatoxin; miCa, mitochondrial Ca^{2+} uniporter; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; OMM, Outer Mitochondrial Membrane; PBL, peripheral blood lymphocytes; PKA, Protein Kinase A; PM, Plasma Membrane; RLM, Rat Liver Mitochondria; PTP, Permeability transition pore; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species; SDH, Succinate Dehydrogenase; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; SMP, Sub-Mitochondrial Particle; SUR, SulfonylUrea Receptor; TSPO, Translocator Protein; VDAC, Voltage-Dependent Anion Channel (porin)

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preparations of isolated mitochondria. In particular, it is by now well recognized that the association of mitochondria with the ER is so close that Mitochondria Associated Membranes (MAM; e.g.: [11–14]) constitute a functional sub-domain providing physical contact between these subcellular compartments. Since MAM are very difficult to eliminate completely, adequate experimental precautions must be taken to ascertain whether the protein(s) under investigation are located in mitochondria (narrowly defined) or in MAM. This is particularly relevant when the proteins in question are abundant in the ER, e.g. in the case of the Ryanodine Receptor, whose presence in mitochondria has been reported [15,16]. Genetic techniques may be useful in this respect [3,17–19]. Using a biochemical approach, the observation that the abundance of a protein in various membranous fractions increases along with that of mitochondrial markers, while markers of contaminating membranes decrease, may be considered good evidence for a mitochondrial location. In the specific case of inner mitochondrial membrane channels, an advantage comes from the ability to conduct patch-clamp experiments involving a limited portion of membrane, which may be visually observed to be the "inflated" inner membrane of a single swollen mitochondrion. Any MAM present would be expected to be associated with the outer membrane, which is largely lost upon swelling and whose remnants can be observed (and avoided) in a large fraction of the objects in the field of view. In support of an inner membrane location of channels routinely observed in patch-clamp experiments on mitoplasts one may invoke a kind of proof *ab absurdo*: if these channels actually resided in OMM/MAM residues, this would imply that a large percentage of seals are actually established on these unstructured remnants rather than on the much vaster, more accessible and carefully targeted surface of the inner membrane. It is doubtful that any high-resistance seal (as distinct from pipette clogging) could actually be established on OMM/MAM residues, and in fact we feel all seals formed on swollen mitoplasts ought to be considered as established on the inner membrane unless proof is provided of the contrary. These considerations do not apply to electrophysiological experiments performed with purified/reconstituted membranes or single proteins or protein complexes. Furthermore, for these approaches the objection can always be raised that the properties of the pores may have been altered during the purification, or that their properties may differ from those *in situ* due the loss of accessory/modulating factors.

In this paper we focus on the latest contributions to mitochondrial channel research from our labs. Only a summary account of recent work on selected other IMM channels is provided. Excellent recent reviews are available [20,21]. Two well-characterized IMM channels are the 107 pS anion-selective channel [22] and the Ca^{2+} channel described by Kirichok et al. [23]. We have recently characterized the "Half-PTP" (HP), an anion channel with a conductance close to one-half that of the PTP [24]. These three channels will be covered first, beginning with the anion-selective ones. Three more IMM channels, K^+-selective pores to which a putative molecular identity label has been attached, will be discussed later.

2. Channels of the inner mitochondrial membrane

2.1. IMAC

The anion-selective 107 pS channel, the first mitochondrial channel to be characterized by patch-clamp [22], has been proposed [25,26] to correspond to the Inner Membrane Anion Channel (IMAC) previously studied with the tools of bioenergetics [27,28]. The identification was based on the comparison between the pharmacological profiles of IMAC as studied by monitoring the swelling of suspended mitochondria and of the channel observed by patch-clamp. Channel activity is promoted by stressful circumstances: alkalinisation of the matrix, Mg^{2+} depletion, depolarization (i.e., matrix-side positive potentials in electrophysiological experiments) and ROS or increasingly oxidizing conditions [22,25,28,29]. The functional role(s) of this channel remain in part to be clarified, but it has been proposed to contribute importantly to the oscillatory behaviour of the $\Delta\Psi_m$ and ROS production by the mitochondrial network in cardiomyocytes subjected to oxidative stress and to ROS-induced further ROS production [20,21,30–33]. IMAC activation by ROS is thought to provide the pathway for superoxide efflux from mitochondria. Interestingly, IMAC/107pS is inhibited or activated by at least some ligands (PK11195, 4′-chlorodiazepam, FGIN-1-27) of the Translocator Protein (TSPO) [25,30,34]. The TSPO, formerly known as the mitochondrial or peripheral benzodiazepine receptor, is a highly conserved OMM protein believed to be fundamental for cholesterol transport and membrane biogenesis [35]. These pharmacological effects have led to the idea that it interacts with and modulates IMAC [30,32].

2.2. The half-PTP (HP)

Our group has studied the Permeability Transition Pore by electrophysiological means (reviews on the PT: [36–43]), showing that its characteristics are compatible with a dimeric structure formed by two cooperating channels [44–46]. The behaviour of the channel studied in rodent mitoplasts indicates that states with both channels open or closed are relatively stable, while a situation with only one of the twin pores open is not: the lonely channel gates rapidly and a transition to a longer-lasting stable arrangement follows in short order. A note of caution: a dimeric organization fits well with models envisioning formation of the PTP by a carrier of the IMM – be it the ANT, PiC [42,47] or another one – but since the molecular identity of the PTP is as uncertain as ever (it has even been proposed to be formed by inorganic polymers [48]), there is no definitive proof of it. The electrophysiological data are also compatible with a monomeric channel capable of entering a conformation with a conductance approximately one-half of maximum.

Stand-alone "hemichannels" (dubbed "HP", for "Half-PTP") had been observed in a few experiments with RLM [44], but too infrequently for a proper characterization. This however becomes possible if mitochondria isolated from the human colon tumour HCT116 cell line are used, since in that system the full-size PTP is observed much less frequently [24]. In patch-clamp experiments on mitoplasts of this origin we have thus been able to characterize electrophysiologically and pharmacologically a channel with a conductance close to one-half that of the PTP. Its relationship to the latter is confirmed not only by the similarity of their biophysical properties, but also by the inverse relationship in the frequency of their appearance in experiments conducted under different conditions or with mitochondria of different origin. A relatively high likelihood of observing the PTP is associated with a low probability of observing the HP, and vice-versa. This implies a very close relationship between the two pores, which, if unrelated, would be expected to be insensitive to the other's presence. It furthermore reinforces the notion that the HP may represent one-half of the PTP dimer. This may be a dissociated monomer or the active member of a pair comprising a permanently inactivated channel. Since HP channels often appear in two's or three's, the HP may also represent a regular PTP couple in which interaction (and hence cooperation) between the constituents has been lost.

The HP channel resides in the inner mitochondrial membrane or at residual contact sites: we recorded HP activity in fluorescent objects (mitoplasts), isolated from a cell line stably transfected with a mitochondria-targeted fluorescent DsRed protein, shown by microscopy to localize only in mitochondria; seals were established on mitochondria swollen beyond the point of outer membrane rupture (mitoplasts); the HP channel co-localized with the 107 pS channel and the PTP, acknowledged markers of this membrane.

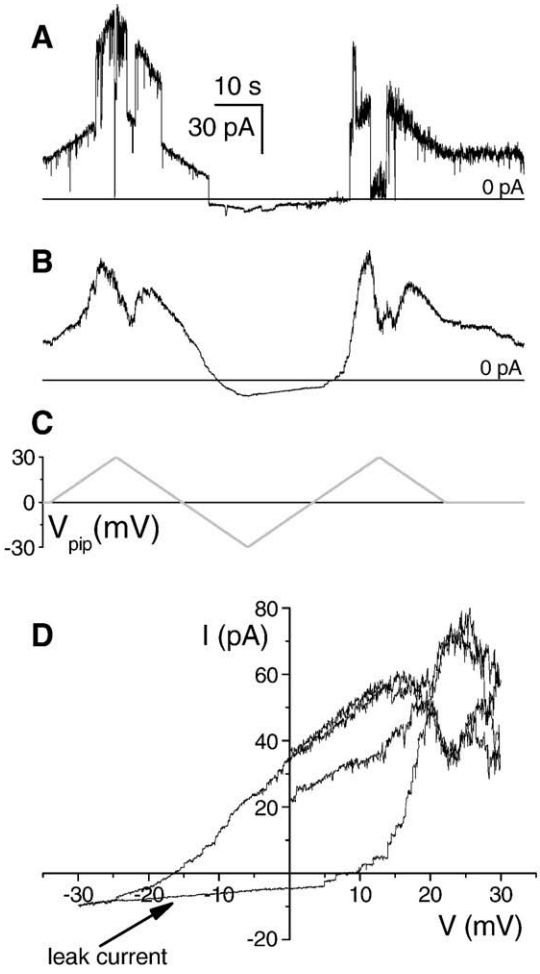

Fig. 1. Reproducibility of HP voltage dependence. (A) A current record obtained by applying the computer-driven voltage protocol shown in panel C to a patch of HCT116 mitoplast membrane. The mitoplast was obtained by allowing mitochondria to swell in the presence of 0.5 mM $CaCl_2$ and 1 mM Pi. The patch contained two active HP channels. Ionic conditions: 0.61 (bath) vs. 0.15 (pipette) M KCl. "Leaks" were not subtracted. (B) The average of 17 consecutive recordings analogous to and including the one shown in panel A, from the same patch. The pattern of panel A is clearly recognizable, indicating that the behaviour of the channels is reproducible. (C) The voltage protocol applied: pipette voltage was ramped between ± 30 mV as shown. The time scale is the same as for panels A and B. (D) I–V plot of the data in panel B.

Based on the properties of these pores, we had suggested that VDAC, the mitochondrial porin, may constitute the ion-conducting portion of the assembly [45,46]. Single-channel recordings on HCT116 mitoplasts confirmed the strong similarities between the HP and VDAC, but uncovered some differences. The HP displays Ca^{2+}-dependence, in the sense that "high" (≥100 μM) Ca^{2+} is generally needed to observe sustained activity. Its conductance, about 400 pS in 150 mM KCl, matches that of VDAC (both channels actually exhibit a range of conductances), but the HP channel is more markedly anion-selective, with a ratio of permeability coefficients $P_{Cl}/P_K \approx 7–18$. Purified VDAC, despite its name, has a lower anion selectivity, with P_{Cl}/P_K estimates ranging only up to about 4. The HP channels displayed voltage sensitivity but with characteristics that differed from case to case. One can identify three patterns. The HP was normally open if no transmembrane voltage was applied, but often showed a VDAC-like tendency to close as the potential was increased in either a positive or negative direction. In most cases closure took place at relatively low (≤40 mV) voltages. In other instances closure occurred more readily at negative than at positive pipette potentials, but in rare cases the opposite was true. The sensitivity to voltage also varied, with some channels responding promptly and at low (<40 mV) voltages, and others requiring prolonged application of higher potentials to close. As exemplified by the records in Fig. 1, repeated applications of standard voltage protocols on the same channel consistently elicited the same response, so that variability cannot be ascribed to stochastic variations. We speculated that post-translational modifications, possibly phosphorylation, of the protein(s) affecting the voltage sensor(s) may account for this variability in voltage-dependence. While, to our knowledge, an asymmetric voltage dependence of porin has not been reported, this discrepancy might be ascribed to modifications intervening upon isolation of the protein. An identification of HP with VDAC has however been ruled out by the observation of the former's activity in MEF cells lacking either VDAC-1, VDAC-2, or both VDAC-1 and -3 [24]. On the other hand, the properties of HP channel are, as expected, similar to those of the PTP. Both are anion-selective, but the PTP can occasionally switch to cationic selectivity for short periods [49,50]. This switch is associated with unstable states. Similarly, in rare cases the HP channel can give rise to cation-selective conductances, as exemplified in Fig. 2.

In an attempt to gain clues as to the molecular nature of the pore, we determined a pharmacological profile which however did not provide definitive information [24]. DIDS and SITS (100–300 μM) inhibited its activity, but other classical inhibitors of anion-selective channels (NPPB, IAA-94, niflumic acid and flufenamic acid) did not. ATP or Mg^{2+} at acid pH were also able to inhibit the channel, as was the reducing agent BHT (Fig. 3). These effects reinforce the link to the PTP, known to be activated by oxidative conditions. On the other hand, Cyclosporin A (CSA) did not block the channel, but for some reason this paramount PT inhibitor is not effective in HCT116 mitochondria [49,51] (for a discussion of the variable effectiveness of CSA see [38]).

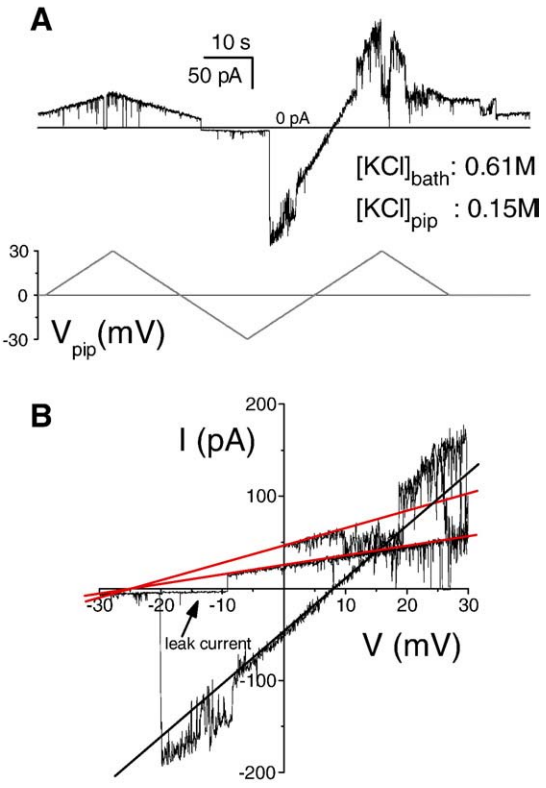

Fig. 2. The HP can transiently adopt cation-selective states. (A) Current record (above) and applied voltage protocol (below) from a representative experiment on an HCT116 mitoplast. Ionic conditions as indicated. An opening to a cation-selective state can be observed, followed by a return to anionic selectivity (positive current at 0 applied voltage at the end of the trace). (B) I–V plot of the data in panel A. While extrapolation of some segments of the record yields a negative x-axis intercept (i.e. a negative reversal potential, corresponding to anionic selectivity), extrapolation of others yields a positive one (cationic selectivity). The leak current was not subtracted.

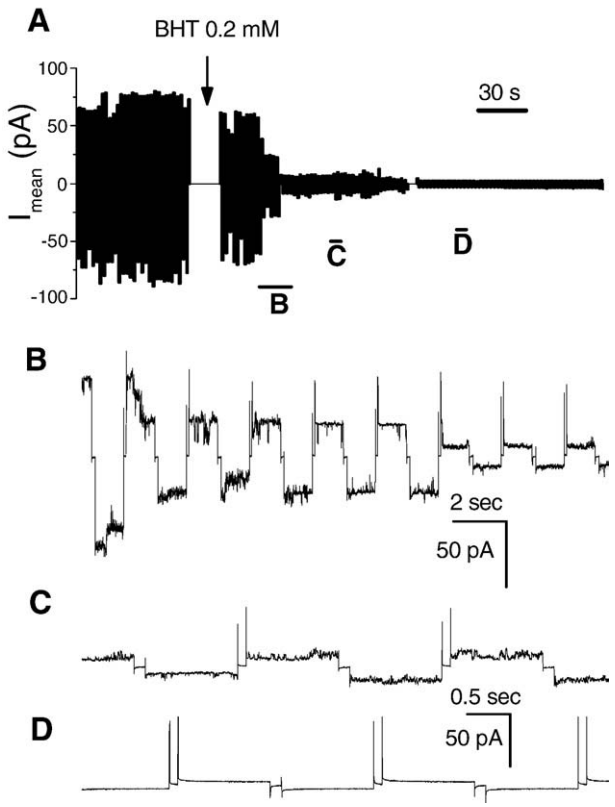

Fig. 3. Inhibition of the HP channel by BHT. In this representative experiment on an HCT116 mitoplast, 20-mV, 1-second voltage pulses of alternating polarity were applied sequentially, with a 0.1 s interval at zero voltage between pulses. (A) A column plot of the mean current flowing in the circuit during each pulse vs. time. Addition of 0.2 mM 3,5-di-t-Butyl-p-HydroxyToluene (BHT) causes closure of the channels, i.e., a decrease of the mean current. (B–D) Current records corresponding as indicated to the plot segments identified by bars and letters in panel A. After the important current decrease shown in panel B the patch still exhibited some residual low-conductance activity (C), which eventually disappeared leaving only the leak current (D).

The reported properties of mitochondrial channels whose molecular identity is known are different from those of the HP. The pores formed *in vitro* by the purified adenine nucleotide translocator, considered until recently to be a core component of the PTP, had conductances in the 300–600 pS range ([KCl] = 100 mM), with substates, were activated by Ca^{2+}, and their conductance was decreased by lowering pH in the 7–5 range. But they closed only at $V>100$ mV, and were slightly cation-selective [52,53]. Purified/reconstituted yeast phosphate carrier [54], brown adipose tissue uncoupling protein [55] and multiple-location intracellular CLIC-4 [56] on the other hand have been reported to form moderately or poorly (CLIC-4) anion-selective pores, but their conductance is clearly lower than that of the HP. Other CLIC channels are much "smaller" [57]. Other possible candidates might be the components of the protein import machinery, whose channel activity upon reconstitution has been described. Isolated Tim22 was reported to be activated at high voltages [58,59], but a recent paper [60] reports that in its native membrane it can be observed at low voltages if challenged with a leader peptide, while it is driven into closed or inactivated state(s) as the voltage is made more negative or positive. Tim23 has been identified [60–63] as the protein responsible for the mitochondrial Multiple Conductance Channel activity, which apparently also exhibited sensitivity to TSPO ligands [34] (see below). Tim22 and Tim23 pores resemble each other and, at least superficially, the PTP. They show evidence for a dimeric structure and their conductance and voltage-dependence may match that of the PTP, but they are cation-selective. Still another candidate is provided by the discovery that connexin-43 hemichannels are present in cardiac mitochondria [64,65]. Connexons are known to be regulated by multiple phosphorylations [66–68], but at least those formed by Cx43 are inactivated by Ca^{2+} and display little selectivity and a considerably lower conductance than the HP channel [69,70]. Other hemi-connexons have suitably larger conductance (e.g.: [71]), but the lack of effect of carbenoxolone, a widely used gap-junction blocker, on HP activity [24] makes an identification of the channel with this type of molecules unlikely.

The properties of the HP channel turned out however to match exactly those of PM "maxi-chloride channels", as described, in particular, by Sabirov et al. [72]. The name indicates an excision- and swelling-activated, voltage-inactivated (set of) pore(s) recorded in the plasma membrane of various cell types (e.g.: [72–74]), which has been proposed to be implicated in the release of ATP and/or glutamate from stressed glial cells [75,76]. This correspondence does not allow decisive progress in the identification of the molecule(s) involved, since the molecular identity of the "maxi" chloride channel is unknown.

2.3. miCa

The properties of the channel described in [23] are broadly consistent with its identification with the long-sought mitochondrial Ca^{2+} uniporter (miCa). For example, it is inhibited by Ru360 and Ruthenium Red, the permeability ranking of divalent cations is the same, and its open probability decreases as the transmembrane potential is lowered. Whole mitochondria have long been known to have a relatively low affinity for Ca^{2+} [77]. Indeed, the observation of a marked increase in intramitochondrial Ca^{2+} in response to cytosolic transients not exceeding 1–3 μM as a peak cytoplasmic average value was surprising and led to the discovery of the "special relationship" between the ER and mitochondria [12,78–82]. In the patch-clamp study inhibition of Na^+ currents by Ca^{2+} led to a 2 nM estimate for the affinity of the uniporter [23]. This difference may be ascribed to the presence, in intact mitochondria, of regulatory elements (e.g., Mg^{2+} ions) which may be absent in an electrophysiological setting, and of Ca^{2+}-exporting mechanisms (e.g. Na^+ or H^+/Ca^{2+} antiporters). Another aspect to be considered is the outward (from the matrix) uniport-mediated Ca^{2+} flow. Suspended mitochondria loaded with Ca^{2+} readily release it via the uniporter if they are depolarized. miCa apparently shows inward rectification not only when Ca^{2+} on the matrix side is low, but also under symmetrical conditions (see Supplementary Fig. 2 in [23]). Whole-mitochondrion I–V curves recorded with "symmetrical" [Ca^{2+}] would clarify whether this rectifying behaviour is compatible with the suspension Ca^{2+} flux data. The abundance of the mitochondrial Ca^{2+} uniporter has been estimated at ≤1 pmol/mg prot. on the basis of La^{3+} binding assays [83]. Kirichok et al. estimated its density at 10–40 channels/μm^2 of IMM surface [23]. Whether the two data are compatible depends on the area of the IMM associated with 1 mg of mitochondrial protein. Not many estimates are available for this parameter. Petronilli and Zoratti [84] obtained a value of 0.92 ± 0.17 μm^2/mg prot. for mouse liver mitochondria, which would translate Kirichok's estimate into roughly 0.2–0.7 pmol/mg prot., a surprisingly close agreement with the binding assay data. It should in any case be considered that the mitochondria used for patch-clamp work were isolated from COS cells, while most of the data on Ca^{2+} transport by populations of isolated mitochondria and related topics have been obtained using RLM.

The molecular nature of miCa is still mysterious. Its properties identify it as a Ca^{2+} channel (rather than, say, a nonselective cation channel). Characteristically, in the complete absence of Ca^{2+} it can conduct Na^+ ions, but very low concentrations of Ca^{2+} essentially block the Na^+ current. This is thought to be due to the high affinity for Ca^{2+} of the selectivity filter, which in PM Ca^{2+} channels consists of four Glu residues. The bound Ca^{2+} can be chased off this binding site by electrostatic repulsion by another incoming divalent cation, but not

by a monovalent ion. Thus one would predict the presence in mitochondria of a protein, or an assembly of proteins, having some homology to known Ca^{2+} channels and tale-telling glutamates (or perhaps aspartates) at the appropriate position. The several mitochondrial proteomic studies published so far have however essentially failed to recognize any mitochondrial channel except the three VDAC isoforms (e.g.: [85–94]). This reflects perhaps a tendency to ascribe to contaminating material any protein not already known to belong specifically to mitochondria (the "eclipse effect"), as well as technical difficulties ascribable to their hydrophobic nature and especially to their low abundance [95,96]. In general, aside from porin, aquaporin and mechanosensitive channels in bacteria, which are relatively abundant, only very few channel proteins have been identified by proteomics.

2.4. Mito KATP

An activity identified as corresponding to that of the ATP-sensitive K^+ channel was described early in the history of mitochondrial electrophysiology [97], but to this day it remains a matter of controversy, with perhaps most of the opinionated favouring its presence in mitochondria, but some articulate opponents (e.g.: [98–102]).

PM K_{ATP} channels are composites of a K^+-conducting portion belonging to the inwardly-rectifying Kir family (Kir6.1 or Kir6.2), and a regulator protein belonging to the ABC transporter class (SUR1 or SUR2; the latter comes in two variants) which largely determines the pharmacological properties of the channel [103]. Unbelievers point out that these putative components have not so far been unequivocally identified as components of the mitochondrial proteome (but see the comment above). Biochemical approaches have produced both affirmative and negative results, and have so far failed to convince skeptics despite a number of reports confirming the presence of K_{ATP} subunits in mitochondria. A detailed review of the literature cannot be given here, but some information is included to provide an idea of the unenviable state of the field. Accounts can be found in recent papers and in reviews [20,21,77,101–107].

Thus, e.g., Bajgar et al. reported that an isolate obtained from brain mitochondria via an ATP affinity column contained two proteins of 55 and 63 kDa apparent MW (assigned to a Kir and SUR subunit, respectively) [108]. Upon incorporation into liposomes this fraction was able to reconstitute K^+ transport with pharmacological characteristics matching those of mtK_{ATP}. Mironova et al. observed the channel in planar bilayer experiments using protein fractions isolated from mitochondria prepared by standard methods [109–111]. In studies of this type it is not always clear whether the precautions adopted were sufficient to prevent contamination artefacts. Suzuki et al. found Kir6.1 in the inner membrane of muscle and liver mitochondria by immunofluorescence and immunogold EM [112]. Lacza et al. used immunogold EM, Western blotting and other methods to conclude that Kir6.1 and Kir6.2 are present in isolated purified mouse heart and brain mitochondria [113,114]. Mitochondria-addressing sequences were putatively identified in the proteins' sequences. Abe's group also identified Kir6.1 in rat brain [115] and kidney [116] mitochondria by immunomicroscopy. Western blot evidence for Kir6.1 in the mitochondria of an amoeba has been obtained [117]. But recent work by the Marbàn group suggests that anti-Kir antibodies may recognize unrelated mitochondrial proteins [118]. Peptides with the MW expected for SUR1 or SUR2 were not detected by Lacza et al. [113]. The commercial anti-SUR2 antibody used recognized instead a 25-kDa protein, and the authors concluded that functional mtK_{ATP} channels are present in heart mitochondria. A glibenclamide-binding protein of similar size, considered to be a SUR-like component of mtK_{ATP}, had also been observed in beef heart SMPs [119]. Both SUR2A and SUR2B were instead detected as higher MW peptides (~50 and ~120 kDa) in rat heart cardiomyocyte mitochondria by Western blotting and immunoelectron microscopy using antibodies made in-house [120].

Evidence in favor of a functional mtK_{ATP} channel comes from electrophysiological observations, obtained via both patch-clamp experiments on mitochondria [97,121] and planar bilayer recordings of activity after fusion of IMM vesicles or incorporation of isolated protein fractions [107,109–111,122–128]. The characteristics of the channels identified, on the basis of pharmacological effects, as mtK_{ATP} in electrophysiological experiments differ somewhat. Inoue et al., patching RLM mitoplasts, described a channel with a single channel conductance (g) of 9–10 pS in 100 mM (pipette)/33 mM (bath) KCl. The I–V relationship was nearly linear, with only a slight outward rectification [97]. The channel of Dhalem et al. (the only other patch-clamp study) exhibited a similar conductance at negative potentials, but rectified more steeply, with g exceeding 80 pS at $V>60$ mV [121]. In early planar bilayer experiments with purified proteins Paucek et al. measured about 30 pS in 1 M KCl [122]. These authors considered this value to be in fair agreement with Inoue's, since the channel was expected to exhibit saturation with increasing [KCl]. Mironova et al. reconstituted protein fractions which produced channels of about 10 pS in symmetrical 100 mM KCl [109–111]. The major conductance observed by Zhang et al. after fusing inner mitochondrial membrane vesicles with the planar bilayer, in symmetrical 150 mM KCl, measured ~56 pS [123], while Bednarczyk et al. observed an essentially ohmic conductance of ~103 pS under the same conditions [107,127,128]. The differences in conductance can however be ascribed at least in large part to a recognized cooperative behaviour of these channels, which appear to often occur in clusters [97,109,111]. Redox sensitivity [123,124,126,129,130] may further contribute to complexity.

In many cases the presence, properties and roles of mtK_{ATP} have been inferred indirectly from experiments employing isolated mitochondria or reconstituted proteoliposomes and pharmacological tools (e.g.: [131–133]). Many of these studies rely on monitoring K^+ fluxes as indirectly measured by swelling of the mitochondria, changes in mitochondrial potential, oxygen consumption or variations in pH or K^+ content as revealed by indicator dyes. This class of experiments, in particular the ones involving measurements of mitochondrial volume, have been criticized in particular by Halestrap and coworkers, who, e.g., did not obtain firm evidence for an increase of mitochondrial volume following application of mtK_{ATP} activator diazoxide [98,101].

All considered, it seems fair to conclude that ROS-, GTP-, GDP-, UDP-, diazoxide-activated, ATP-, ADP-, Mg^{2+}-, NO-, quinine-, 5-HD-, glybenclamide-inhibited K^+-selective channels are likely to be present or can be formed in the IMM of various tissues and cells.

The molecular composition of the mtK_{ATP} channel remains however an open question. Information about this key point was sought by comparing the properties and pharmacology of an activity assigned to mtK_{ATP} with those of surface channels of defined molecular composition formed by Kir and SUR subunits produced in HEK293 cells from transfected cDNAs [134,135]. In both studies activity of heterologously expressed, surface-located Kir-SUR combinations (or of native cardiac myocyte channels) was assessed by whole-cell patch-clamping, while activity of the mtK_{ATP} counterparts was assessed indirectly by monitoring the fluorescence of mitochondrial flavine nucleotides. This latter method reports on the redox state of these groups, which is a function of the $\Delta\tilde{\mu}_H$ maintained across the IMM. Thus, it reports the degree of depolarization or of uncoupling of the mitochondria themselves. Opening of mtK_{ATP} channels would indeed be expected to lead to mitochondrial depolarization. The method was adopted on the basis of the observation [136] that diazoxide – which opens mitochondrial but not plasma membrane K_{ATP} channels in cardiac myocytes [137,138] – induced a decrease in the fluorescence of flavin nucleotides. Its validity has later been challenged [139]. These comparisons along with imaging studies led to the conclusion that mtK_{ATP} in the heart is "distinct from surface K_{ATP}

channels at a molecular level", i.e., it is not composed of Kir6.2 and SUR2A, the subunits of the PM K_{ATP} in these cells [134]. A match was found instead between the pharmacological profiles of fluorescence variations linked to mtK_{ATP} activity in myocytes and of current conduction by the Kir6.1/SUR1 combination [135]. In view of other data, however, the identification was considered tentative by the authors themselves.

In 2004 Ardehali et al. [140], building on previous results showing that diazoxide and 5-HD affected succinic dehydrogenase activity as well as mtK_{ATP} [141], reported the purification of a complex of 5 mitochondrial proteins, including succinate dehydrogenase. Upon reconstitution in planar lipid bilayers this preparation produced channels with g ~200 pS in 500 vs. ~30 mM KCl. K^+ selectivity was not pronounced, but this was tentatively ascribed to the presence of –SH reducing agents. Importantly, the activity (and the permeability of proteoliposomes to K^+) responded to modulators in the manner expected if it were due to mtK_{ATP}. Furthermore SDH inhibitors, but not ANT inhibitors, affected both permeability to K^+ of liposomes and channel activity in planar membranes. Malonate, a well-known SDH inhibitor, was recently confirmed to activate mtK_{ATP} (monitored as mitochondrial swelling) [142].

mtK_{ATP} presumably participates in the regulation of mitochondrial ionic homeostasis, but the real reason so much interest is concentrated on this particular mitochondrial channel is the role it may play in ischemic preconditioning (IP). IP consists of brief sub-lethal ischemic periods that are capable of protecting against subsequent massive ischemia [143]. The discovery of "chemical preconditioning" by K_{ATP} openers [144] and its subsequent attribution to mitochondrial K_{ATP} [132,137,145] spawned a whole field of research. The reader is referred to several excellent recent reviews for a complete overview [101,102,105,106,146–157]. The phenomenon has been studied most in the context of cardioprotection, but it has also been observed for skeletal muscle (e.g.: [158,159]) and other organs. It goes without saying that this is also a ground of dispute, with skeptics pointing out the multiplicity of other effects of mtK_{ATP} agonists and antagonists. The mechanism of preconditioning has not been established yet, but clearly it is a complicated process involving various cellular signalling pathways, with a major role played by PKCε, as well as possibly other mitochondrial channels ($mtBK_{Ca}$, PTP, hemi-connexon 43) besides mtK_{ATP}. It might actually be more appropriate to consider a role for mitochondrial K^+ influx rather than for mtK_{ATP} activation, since opening of $mtBK_{Ca}$ also seems to result in protection (see below). A key facet of the mechanism seems to be ROS (and RNS [154,160]) production at mitochondria [101,154,161,162]. In one scheme the end effect of preconditioning is to reduce oxidative stress during ischemia/reperfusion, i.e., to decrease the extent of Permeability Transition in the infarcted organ (Ca^{2+}-elicited PTP opening has long been known to be strongly potentiated by thiol oxidation; see e.g. [163]). This end effect is thought to be the only apparently paradoxical consequence of a (more limited) ROS production during preconditioning (e.g. [101] and refs therein). According to one view, these would have the effect – via still poorly defined ways – of activating PKCε (e.g. [164–166]) and other kinases, whose signalling would converge in the end in the phosphorylation and inhibition of GSK3β. The latter has been reported to act at mitochondria to facilitate opening of the PTP and cell death [167–170]. A point that has not been adequately clarified is how mtK_{ATP} opening should lead to increased ROS production. In fact some results suggest that the channel may be not upstream but rather downstream of ROS generation caused by other phenomena (in particular, when diazoxide is used as a preconditioning agent, by inhibition of SDH) [101,139,171,172]. Another view is that mtK_{ATP} opening is by itself responsible for the protecting mechanism (e.g.: [173]). This relates to the observation that mild uncoupling by various means before ischemia is protective (reviewed in [101]). Opening of mtK_{ATP} would be expected to cause a decrease of $\Delta\psi_m$. The mechanism of signal transduction leading to protection is however at least as obscure in this scheme as in the one envisioning ROS.

In conclusion, there is as yet no general consensus on the role of mtK_{ATP} in preconditioning protection.

2.5. Ca^{2+}-activated K^+ channels

Given the relevance of Ca^{2+} in mitochondrial physiology, and given the impact Ca^{2+} handling by mitochondria has on cellular processes, it is not surprising that the IMM has been found to contain Ca^{2+}-activated channels. One special case of such a channel is the PTP, briefly mentioned above. Mitochondrial editions of two Ca^{2+}-dependent K^+ channels present and studied in the PM have been described. One is $mtBK_{Ca}$ ($K_{Ca}1.1$ [174]), observed by direct patch-clamping of mitoplasts [175–178] as well as in planar lipid bilayer experiments [179]. Evidence has also been provided by Western blot, electron microscopy and immunofluorescence microscopy [176,179,180]. The identity has been confirmed by pharmacological studies employing selective toxins, and the presence of this channel in mitochondria appears to be less controversial than that of K_{ATP}, even though doubts have been voiced, mainly on grounds of possible contamination by other membranes [101]. The channel is activated by Ca^{2+} in the μM range and also in other respects seems to conform to the PM channel model (for which see, e.g.: [181–184]). The latter channel, called "Big" because of its 100–300 pS conductance, is known to be a tetramer of α subunits (occurring in splice variants) crossing the membrane 7 (rather than 6 as in K_V channels) times, with the N terminus outside and a large C-terminus domain in the cytoplasm. This domain contains the Ca^{2+}-binding "bowl" and protein–protein interaction motifs ("leucine zippers") and phosphorylation sites known to be used by PKA and other kinases. BK_{Ca}'s are also voltage-dependent due to the presence of a positively charged S4 segment, the movement of which induces opening upon depolarization. Based on the observed voltage-dependence (opening favored by matrix-positive, unphysiological, potentials), the orientation of $mtBK_{Ca}$ in the IMM can thus be deduced to be the same as in the PM. Four (plus splice variants) membrane-inserted, N-terminal-interacting auxiliary β subunits are known, and account for a certain organ-dependent variability in the channels' properties [185]. The β1 subunit has been proposed to be associated with $mtBK_{Ca}$ in cardiac mitochondria [177], while a recent publication reports that the mitochondrial channel in neurons specifically comprises the β4 subunit (KCNMB4) [180].

BK_{Ca} channels of the PM are very useful negative feedback tools as they can counteract depolarization and Ca^{2+} entry via voltage-dependent Ca^{2+} channels by opening and promoting (re)polarization (e.g. [186]). $mtBK_{Ca}$ is believed to carry out a similar feedback regulatory role linking matrix $[Ca^{2+}]$ to IMM K^+ permeability and mitochondrial volume [176]. Recent studies have found evidence for a role in acute hypoxia sensing (hypoxia increases Po) and of a functional interaction with the PTP [178,187].

As in the case of mtK_{ATP}, much interest concentrates on this pore because of the protective role its opening has been reported to afford against I/R damage to the heart and other organs [176,177,188–195]. In this case any effects may well be attributed to a mitochondrial channel, since BK_{Ca} seems not to be expressed in the PM of cardiomyocytes. Since both mtK_{ATP} and $mtBK_{Ca}$ activation lead to an increase in the K^+ permeability of the IMM and thus to an increased K^+ influx, the findings that activation of each protects from damage reinforce the case for a role by both or either (e.g. [196]). The mechanism of protection would be predicted to be the same. Coherently, $mtBK_{Ca}$ activation is reported to lead to an increased rate of respiration and to increased production of ROS by mitochondria, which would be a key factor in delayed protection (but a ROS decrease has also been reported; e.g.: [193]). Again, the matter is

controversial. Much of the evidence supporting such a protective role is based on the finding that NS1619, a BK_{Ca} activator, induces preconditioning-like protection in the heart. Like for diazoxide and 5-HD in the case of mtK_{ATP}, there is however concern that this may well be due to other, $mtBK_{Ca}$-independent effects such as induction of ROS generation (by an unidentified mechanism) [197–200]. Analogous concerns have not been voiced with regard to the converse protection-abolishing effect of BK_{Ca} blockers, most often paxilline. Again, why ROS production should be increased upon increasing IMM permeability to K^+ remains unclear. Recently two novel BK_{Ca} agonists, NS11021 [201,202] and diCl-DHAA [192,203] have been used, producing much the same protective effects. A new blocker, HMIMP, has also become available [204].

The other two families of Ca^{2+}-activated K^+ channels, as defined on the basis of channel conductance, are the small (2–25 pS) conductance SK_{Ca} [205,206] and the intermediate (25–100 pS) conductance IK_{Ca} ($K_{Ca}3.1$ [174]) [207–211]. There is no information on the presence of the former in mitochondria, but the latter has recently been discovered in the inner membrane of human colon tumour (HCT116) mitochondria (De Marchi et al., submitted).

Plasma membrane IK_{Ca} (revs.: [212,213]) is present mainly in blood and in epi- and endothelial cells. In the latter, it aids fluid secretion by providing a pathway for K^+ efflux. Along with $K_V1.3$, it is the major K^+ channel in subpopulations of activated T cells (for details see [213,214]). Like the other Ca^{2+}-activated K^+ channels, it provides a feedback mechanism whereby cytoplasmic Ca^{2+} increase results in a hyperpolarizing response counteracting depolarization associated with Ca^{2+} influx and sustaining Ca^{2+} entry. This circuit is important for progression through the cell cycle. Given the widespread presence of the channel in cancer cells, it is therefore under consideration as a target for anti-tumoural as well as immunosuppressive intervention (e.g.: [215]). Ca^{2+} sensitivity is due to the tight, Ca^{2+}-independent association of calmodulin at the C terminus (e.g.: [216]). The K_D for Ca^{2+} binding to the calmodulin EF hands is ~300 nM, thus any significant increase above the resting level of approximately 1–200 nM Ca^{2+} causes channel opening. There is, instead, no dependence of the open probability on voltage: like K_V channels, IK_{Ca} is formed by 4 subunits each spanning the membrane six times, but the S4 segment lacks the necessary charged residues. The channel is selectively inhibited by maurotoxin and by some triarylmethane derivatives, of which the most powerful (tens of nM range) and useful are clotrimazole and TRAM-34 [215,217].

In mitochondria the protein is present at low levels, but it can be clearly detected in Western blots. In keeping with its low abundance, only a few copies of the channel are observed by patch-clamp in any given membrane patch. The biophysical and pharmacological properties of the mitochondrial IK_{Ca} are indistinguishable from those of the IK_{Ca} activity recorded in the PM of the same cells, which in turn is analogous to that previously described in a number of other cells.

PM IK_{Ca} is very sensitive to $[Ca^{2+}]$, set to open as soon as its levels rise above resting. The presence of the same channel in the IMM and its orientation imply the presence of calmodulin in the mitochondrial matrix, in agreement with reports of calmodulin in these organelles [218,219]. Mitochondria have also been reported to contain CaM-binding proteins [220–225]. Thus, the presence and functions of this paradigmatic Ca^{2+}-sensitivity conferring protein in mitochondria may well be a rewarding field of investigation.

Just how widespread $mtIK_{Ca}$ is needs to be established. If it were present in most untransformed cell types, it could be considered to be a constitutive component of the mitochondrial system of K^+ "uniporters" [21,43,77,131,226]. At the cellular level, $mtIK_{Ca}$ may be expected to have protective roles similar to those proposed for mtK_{ATP} and $mtBK_{Ca}$ (see above). On the other hand, it might instead – or also – have a role in cell death, in analogy to the function recently discovered for $K_V1.3$ in lymphocyte mitochondria (see below).

2.6. $mtK_V1.3$

We have recently identified another potassium-selective channel, $K_V1.3$ [227], in the inner mitochondrial membrane of lymphocytes [228]. This is a member of the much-studied voltage-dependent *Shaker*-like family [212,229–231]. Briefly, this large family of gene products forms tetrameric ion-conducting assemblies, generally associated with a tetramer of one of a number of β subunits. The key feature, besides K^+ selectivity, is voltage dependence, consisting in rapid depolarization-induced opening, followed by inactivation.

$K_V1.3$ is the predominant type of voltage-gated K_V channel expressed in the plasma membrane in human lymphocytes (e.g.: [232]). Activation of $K_V1.3$ channels in T cells is a key event in proliferation, so that specific inhibitors of $K_V1.3$ have a strong immunosupressant effect [214]. Suppression of $K_V1.3$ activity upon stimulation of the CD95 cell death receptor or treatment with C_6-ceramide has been linked to the early, induction phase of apoptosis in T lymphocytes [233,234]. While early inactivation of plasma membrane $K_V1.3$ seems to be important in the initiation of apoptosis by some stimuli, e.g., C_6-ceramide or CD95 [233–235], other stimuli, e.g., the cytostatic drug Actinomycin D, seem to activate the channel in a later phase of apoptosis, signalling execution [236]. An apoptosis-induced increase of plasma membrane $K_V1.3$ activity has been observed to be downstream of caspase-8 activation [237]. These studies leave little doubt that the $K_V1.3$ is deeply involved in the regulation of apoptosis, but genetic proof was required for a full demonstration.

As a genetic model, we used CTLL-2 lymphocytes, known to be deficient for $K_V1.3$ [238] (CTLL-2/pJK), and stably transfected these cells with Kv1.3 (CTLL-2/Kv1.3). The biophysical characteristics of $K_V1.3$ in the plasma membrane of CTLL-2/$K_V1.3$ cells were identical to those of endogenous $K_V1.3$ in Jurkat T lymphocytes, indicating the suitability of this genetic model to analyze the function of $K_V1.3$. To analyze the functional importance of $K_V1.3$ for the induction of apoptosis, we incubated $K_V1.3$-deficient CTLL-2 cells, $K_V1.3$-reconstituted CTLL-2/$K_V1.3$, Jurkat lymphocytes or activated human peripheral blood lymphocytes (PBL) with tumour necrosis factor α (TNFα), staurosporine, sphingomyelinase, C_6-ceramide or Actinomycin D [236,238]. Analogous experiments were performed using siRNA targeting $K_V1.3$. $K_V1.3$-expressing cells responded within 12 h with DNA fragmentation, cytochrome *c* release, mitochondrial depolarization and morphological alterations typical of apoptosis, whereas $K_V1.3$-deficient CTLL-2/pJK cells were resistant. Fig. 4 illustrates significant differences in C_6-ceramide-induced cytochrome *c* release between CTLL-2/pJK and CTLL-2/$K_V1.3$ cells. Dihydro-C_2-ceramide, an inactive relative of C_2- and C_6-ceramides, did not induce release of

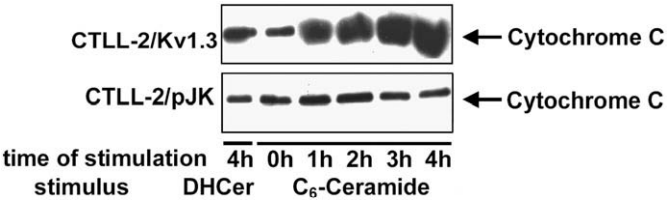

Fig. 4. Treatment of CTLL-2/$K_V1.3$ cells with 20 μM C_6-ceramide from a stock solution in DMSO results in cytochrome *c* release while cells lacking $K_V1.3$ fail to respond to apoptotic stimuli. Cytosolic fractions of whole cell lysates obtained following the indicated treatments were loaded on SDS-PAGE and Western blotted with anti-cytochrome *c* antibodies. To measure the release of mitochondrial cytochrome *c*, cells were washed after stimulation in cold HEPES/Saline, incubated for 30 min at 4 °C in 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM succinate-KOH (pH 7.3), 10 μM cytochalasin B, and 10 μg/ml A/L, and finally Dounce-homogenized. Samples were centrifuged, proteins (20 μg) in the supernatants were separated on 15% SDS-PAGE and blotted onto a nitrocellulose membrane. Western blots were analyzed using a monoclonal mouse anti-cytochrome *c* antibody (clone 7H8.2C12, BD Pharmingen, San Diego, CA) and the Tropix ECL system.

cytochrome *c* in either system. These observations, as well as the fact that prolonged (24–36 h) incubation of CTLL-2/pJK cells with these stimuli eventually resulted in death, suggested that $K_V1.3$ functions upstream of cytochrome *c* release and may be involved in an amplification loop such as the mitochondrial pathway mediating apoptosis. These data prompted us to investigate the subcellular localization of $K_V1.3$.

Evidence was found for a previously undescribed mitochondrial localization of the $K_V1.3$ in genetically non-manipulated lymphocytes [228]. The molecular identity of the channel was assessed by comparing mitochondria isolated from the CTLL-2 line with those from CTLL-2 cells stably transfected with $K_V1.3$. The channel was shown to be present in mitochondria by Western blot, immunogold labelling and FACS analysis. Recent work suggests that also other K_V channels, in particular $K_V1.5$, might be expressed in mitochondria [240]. Most importantly, patch-clamp on mitoplasts indicated the presence of an activity compatible with that of the plasma membrane $K_V1.3$ in mitochondria isolated from Jurkat and CTLL-2/$K_V1.3$ but not in those from CTLL-2/pJK. Fig. 5 shows co-localization of the inner membrane hallmark 107 pS channel and of $K_V1.3$ in mitochondria isolated from Jurkat cells. Both channels were identified on the basis of their biophysical and pharmacological properties. In particular, the slope conductance of mt$K_V1.3$ was approximately 25 pS, a weak rectification was observed at negative potentials in symmetrical high K^+ solution, as expected for $K_V1.3$ [241] and the channel was inhibited from the periplasmic (non-matrix) side by Margatoxin (MgTx), a specific inhibitor of $K_V1.3$. The observed activity displayed potassium selectivity and was only slightly voltage-dependent [228]. This last feature differs from the behaviour of plasma membrane $K_V1.3$, indicating that the channel is differently modulated in mitochondria. In fact, the channel seems to be active even at the very negative resting mitochondrial potential, i.e. at approximately −180 mV, and is likely to be at least partially responsible for the much studied "basal" K^+ conductance in these organelles [77]. Activity of the channel could not be observed directly by patch-clamp at those high voltages, but specific inhibition of mt$K_V1.3$ by MgTx induces hyperpolarization as assessed by indicator dyes. The electrochemical gradient for K^+ predicts that K^+ should enter the matrix through an IMM-located potassium channel. If influx becomes inhibited, a hyperpolarization is expected. Hyperpolarization in turn results in the reduction of respiratory chain components such as Fe/S centers, cytochromes and the ubiquinone pool, and in enhanced production of reactive oxygen species (ROS) (e.g.: [20,162]). In agreement, MgTx addition to isolated mitochondria in suspension resulted in ROS production as illustrated in Fig. 6 (lower trace). ROS are able to oxidize thiol groups and thus to elicit mitochondrial depolarization by activation of the PTP [36,242,243]. PTP opening and consequent $\Delta\psi_m$ decrease downstream of transient mitochondrial hyperpolarization and/or increase in ROS

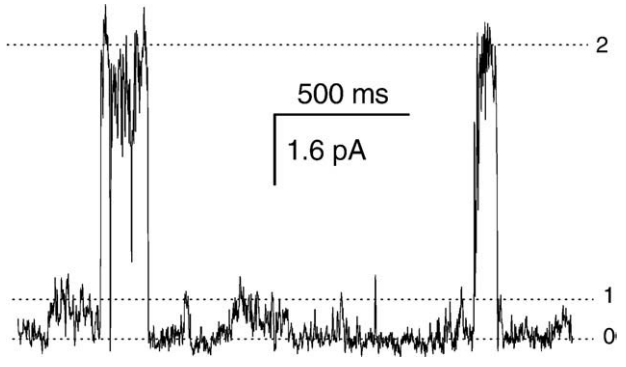

Fig. 5. Co-presence of mt$K_V1.3$ and the 107 pS channel in the same patch of a Jurkat mitoplast. Level 1: current conducted by open mt$K_V1.3$. Level 2: 107 pS channel. Medium: symmetrical 134 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/KOH (pH 7.4), 10 mM EGTA/KOH. Voltage: 60 mV. Sampling frequency: 10 kHz. Filter: 200 Hz.

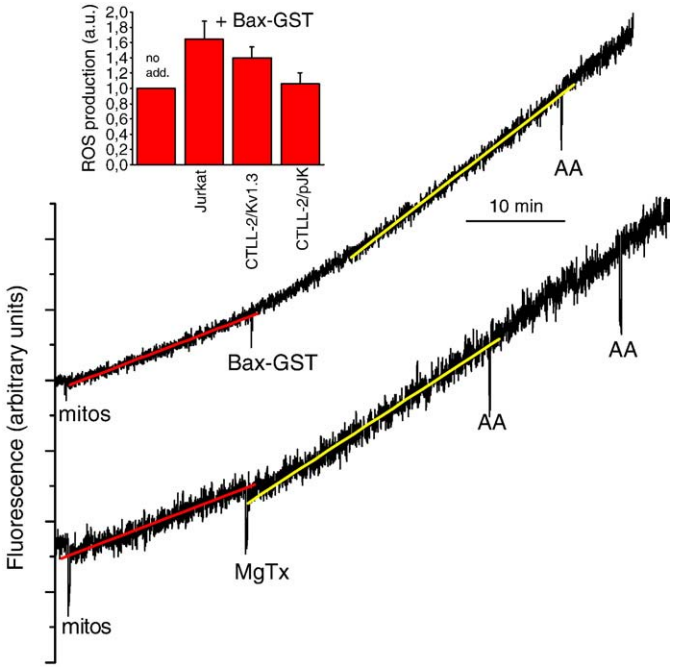

Fig. 6. Increased production of ROS by MgTx- or Bax-treated Jurkat mitochondria. ROS production by mitochondria was monitored as the generation of H_2O_2 in the presence of Superoxide Dismutase using the Amplex Red fluorimetric assay (Molecular Probes) according to the manufacturer's instructions. Mitochondria (corresponding to 1×10^6 cells) were suspended in a stirred, thermostated (25 °C) 2 ml cuvette in a Perkin Elmer LS50B fluorimeter in 50 mM KCl, 170 mM sucrose, 20 mM HEPES/K^+ (pH 7.4), 2 mM $MgCl_2$, 5 mM EGTA, 5 mM succinate, 1 U/ml Superoxide Dismutase (0.1 mg/prot/ml), 15 μg/ml Horseradish Peroxidase and 10 μM Amplex Red. Fluorescence was sampled at 100 min^{-1}. The data were normalized so as to give the same slope of the linear fit of the first segment (before any addition) and to eliminate variations due to the use of different amounts of mitochondria (because of different preparation purity levels) and/or different ROS detection efficiency in the experiments. The relative rate of H_2O_2 production after additions was evaluated from the slope of the fit of the linear portion of the relevant trace segments, as illustrated. Inset: averages of the normalized slopes (mean ± SD; $n=3$) of change in fluorescence upon GST-Bax treatment in similar experiments with mitochondria isolated from the indicated cell types (differences are significant, $p<0.05$, for Kv1.3-positive mitochondria, but not for CTLL-2/pJK mitochondria).

production has been reported in several studies employing drugs or Ca^{2+}-overload to induce apoptosis (e.g.: [244–246]). In our case, MgTx-induced hyperpolarization was indeed followed by CSA-sensitive depolarization, indicating that PTP opening was eventually induced by MgTx [239]. Although PTP opening often requires Ca^{2+} accumulation in the matrix, some inducers, including SH reagents, can function at very low external Ca^{2+}. ROS have also been shown to oxidize cardiolipin resulting in the release of cytochrome *c* from the inner mitochondrial membrane [247,248]. Independently of whether the PTP is related or not to cytochrome *c* release, $K_V1.3$ has been shown to be crucial for cytochrome *c* release when mitochondria were challenged with MgTx (Fig. 7). Thus according to our model inhibition of $K_V1.3$ by specific drugs (MgTx and Shk, another inhibitor of $K_V1.3$) induces ROS production which finally results in cytochrome *c* release from $K_V1.3$-positive mitochondria.

The work summarized above left open the question whether the release of cytochrome *c* by $K_V1.3$ inhibitors has any physiological significance. In other words: is there any physiological stimulus that could inhibit mitochondrial $K_V1.3$ and thereby induce cytochrome *c* release during apoptosis? MgTx and Shk are positively charged peptides that interact with negatively charged residues in the $K_V1.3$ protein vestibule to block the pore. All toxins that block $K_V1.3$ contain a lysine which is critical for the interaction with $K_V1.3$ [249,250]. A model of the structure of the membrane-integrated Bax monomer indicates that at least amino acids 127 and 128, located between the

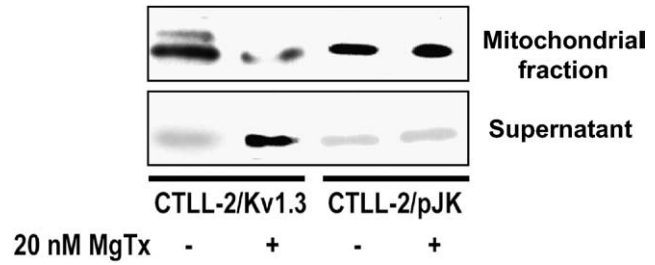

Fig. 7. Margatoxin induces cytochrome *c* release from K_V1.3-expressing mitochondria. Isolated and purified CTLL-2/Kv1.3 or CTLL-2/pJK mitochondria were incubated with 20 nM MgTx for 30 min and the release of cytochrome *c* was determined. The Western blots show the cytochrome *c* content in mitochondria prior and after treatment with MgTx as well as the corresponding amount of cytochrome *c* in the supernatant of the same mitochondrial preparations. To determine cytochrome *c* release from isolated mitochondria, cells were incubated for 30 min at 4 °C in 0.3 M sucrose, 10 mM TES (pH 7.4), and 0.5 mM EGTA and Dounce-homogenized. Nuclei and unbroken cells were pelleted by centrifugation for 5 min at 600 ×*g* and 4 °C. Supernatants were centrifuged at 6000 ×*g* for 10 min at 4 °C, and mitochondria were further purified using 10-min Percoll gradient centrifugation at 4 °C (60%, 30%, 18% Percoll in the buffer as above, 8500 ×*g*). Mitochondria at the interface between the 30% and 60% layers were collected, washed twice, and resuspended in 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 μg/ml A/L, 2 mM ATP, 10 mM phosphocreatine, 5 mM succinate, and 50 μg/ml creatine kinase (buffer 1). Mitochondria were then incubated for 30 min on ice with 20 nM MgTx, respectively, and centrifuged; supernatants were discarded and the mitochondria were resuspended in 37 °C prewarmed buffer 1. Samples were incubated for 5 min at 37 °C and the reaction was terminated by addition of one volume ice-cold buffer 1, centrifugation at 14,000 rpm for 10 min at 4 °C and addition of SDS-sample buffer. The samples were analyzed for cytochrome *c* release by Western blotting.

5th and 6th helices, protrude from the outer mitochondrial membrane into the intermembrane space [251]. Interestingly, the amino acid in position 128 is a highly conserved, positively charged lysine, which may mimic the action of the critical lysine in K_V1.3-blocking toxins by binding to the ring of 4 aspartate residues of the channel vestibule. As mentioned above, the channel vestibule faces the intermembrane space. As we expected, Bax turned out to inhibit K_V1.3. Immunoprecipitation and patch-clamp data showed that Bax interacts with the vestibule region of K_V1.3 [239]. Like MgTx, Bax induced an increase of ROS production by isolated mitochondria (Fig. 6, upper trace and inset). Our results identify mtK_V1.3 as a novel target for Bax and indicate that mtK_V1.3 is required for induction of apoptosis by Bax in lymphocytes. The role of mtK_V1.3 was underscored in experiments in which incubation of isolated mitochondria with recombinant Bax triggered apoptotic alterations only in mitochondria expressing mtK_V1.3. The amino acid residue K128 in Bax seems indeed to be particularly important for the interaction of Bax with mtK_V1.3, since mutation of this lysine to glutamic acid abrogated inhibition of K_V1.3 by Bax and the pro-apoptotic activity of Bax [239]. Our data altogether suggest that Bax and the toxins ShK and MgTx act on K_V1.3 in a functionally similar way.

3. Conclusion and perspectives

Our knowledge of mitochondrial IMM channels is advancing, albeit slowly. Much remains to be done. The molecular identities of the 107pS channel, of the HP and the PTP, and of the miCa remain open questions. The controversy about the existence of mtK_{ATP}, mtBK_{Ca} and their role in preconditioning is likely to continue for some time. Obtaining answers will probably require a systematic investigation combining inventive genetic, biochemical and electrophysiological approaches. The presence of mtK_V1.3 and mtIK_{Ca} channels in the mitochondria of cell types other than the ones in which they were discovered needs to be considered. It is possible that other channels of the K_V and K_{Ca} families (and others?) might comprise a mitochondrial population. Preconditioning and related controversies aside, the role of these various channels in mitochondrial and cellular physiology definitely deserves further investigation, with an eye to possible pharmacological exploitation.

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