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An investigation of the occurrence and properties of the mitochondrial intermediate-conductance Ca^{2+}-activated K^+ channel $mtK_{Ca}3.1$

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ABSTRACT

The mitochondrial intermediate-conductance Ca^{2+}-activated K^+ channel $mtK_{Ca}3.1$ has recently been discovered in the HCT116 colon tumor-derived cell line, which expresses relatively high levels of this protein also in the plasma membrane. Electrophysiological recordings revealed that the channel can exhibit different conductance states and kinetic modes, which we tentatively ascribe to post-translational modifications. To verify whether the localization of this channel in mitochondria might be a peculiarity of these cells or a more widespread feature we have checked for the presence of $mtK_{Ca}3.1$ in a few other cell lines using biochemical and electrophysiological approaches. It turned out to be present at least in some of the cells investigated. Functional assays explored the possibility that $mtK_{Ca}3.1$ might be involved in cell proliferation or play a role similar to that of the *Shaker*-type $K_V1.3$ channel in lymphocytes, which interacts with outer mitochondrial membrane-inserted Bax thereby promoting apoptosis (Szabò, I. et al., Proc. Natl. Acad Sci. USA 105 (2008) 14861–14866). A specific $K_{Ca}3.1$ inhibitor however did not have any detectable effect on cell proliferation or death.

1. Introduction

Over the past fifteen years or so the operational concept of monovalent ion "leaks" through the inner mitochondrial membrane (IMM) has been replaced by firm evidence for the presence of a multi-component, regulated transport machinery for both cations and anions [1–6]. K^+ is the major intracellular cation, and a relevant mitochondrial K^+ cycle consisting of electrophoretic, channel- and "leak"-mediated influx, and electroneutral efflux via K^+/H^+ exchange is in place. The K^+-selective pores reported to be present in the IMM so far are five, all of them mitochondrial counterparts of channels known to be present in the cellular membrane: mtK_{ATP} [3,7–9], whose presence in mitochondria is still debated (for a recent discussion see [5]), *Shaker*-type $K_V1.3$ [10–12], twin-pore TASK-3 [13], and two (out of the three known) types of Ca^{2+}-activated K^+ channels: $K_{Ca}1.1$ (BK_{Ca}) and $K_{Ca}3.1$ (IK_{Ca}) [14–20].

The Ca^{2+}-activated, high-conductance $mtBK_{Ca}$ ($K_{Ca}1.1$) was first observed in mitoplasts from a glioma cell line [14], and it has been found also in the IMM of cardiomyocytes [15] and in skeletal muscle [16], liver [17] and brain [18] mitochondria. Its activity has furthermore recently been observed in bilayer experiments with IMM preparations from potato mitochondria [19]. Recently, our group has characterised the intermediate-conductance $K_{Ca}3.1$ in the IMM of HCT116 cells [20]. This paper reports further progress in the study of this latter channel.

Abbreviations: BK_{Ca}, big conductance Ca^{2+}-activated K^+-channel ($K_{Ca}1.1$); Clotrimazole, 1-[(2-chlorophenyl)-diphenyl-methyl]imidazole; CsA, Cyclosporin A; DC-EBIO, 5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one; DMEM, Dulbecco's modified Eagle medium; ER, endoplasmatic reticulum; GST, glutathione-S-transferase; HBSS, Hank's balanced saline solution; HCT116, human colon tumor 116; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel ($K_{Ca}3.1$); IMM, inner mitochondrial membrane; I/R, ischemia/reperfusion; K_{ATP}, ATP-sensitive K^+ channel; MAM, mitochondria-associated membranes; MDR, multiple drug resistance; MEF, mouse embryonic fibroblast; MgTx, margatoxin; MPTP, mitochondrial permeability transition pore; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OMM, outer mitochondrial membrane; PACS, phosphofurin acidic cluster sorting; PBS, phosphate buffered saline; PKA, protein kinase A; PKC, protein kinase C; PM, plasma membrane; PMCA, plasma membrane Ca^{2+}-ATPase; RISK, reperfusion injury salvage kinases; RLM, rat liver mitochondria; ROS, reactive oxygen species; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase; ShK, stichodactyla toxin; TASK-3, TWIK-related acid-sensitive K^+ channel ($K_{2P}9.1$); TRAM-34, [1-(2-chlorophenyl)diphenyl)methyl]-1*H*-pyrazole; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

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Given the important and nearly ubiquitous role of Ca^{2+} in cellular signalling, it is not surprising that cells should have a variety of Ca^{2+}-activated K^+ channels. Besides $K_{Ca}1.1$ (reviews: [21,22]) and $K_{Ca}3.1$ (rev.: [22–24]), these include the $K_{Ca}2$ family of three members, also known as SK_{Ca} because of their lower conductance (rev.: [25]). Two major functions of these channels are to link cytoplasmic Ca^{2+} levels and PM potential – a negative feedback mechanism in excitable cells – and to provide a Ca^{2+}-dependent K^+ permeation pathway, important in secretory epithelia.

$K_{Ca}1.1$ (also called Slo, Slo1, and BK or maxi K channel due to its large conductance of about 260 pS) forms tetrameric complexes composed of four ion-conducting α subunits, each with 7 transmembrane segments and an intracellular domain which acts as Ca^{2+} sensor, and four regulatory β subunits (four variants of which exist). Besides Ca^{2+}, it is modulated by voltage and mechanical stress. It is expressed by a wide variety of cells, including neurons and myocytes, and has pleiotropic functions. It can respond to transient Ca^{2+} increases ("sparks") originated by opening of PM Ca^{2+} channels or sarcoplasmic reticulum ryanodine receptors, and it has been observed to form clusters optimally positioned for such local responses, for which it seems to have been particularly designed by evolution since it responds to $[Ca^{2+}]$ in the μM range.

$K_{Ca}2.1/2/3$ (SK) differs in most respects, exhibiting low single-channel conductances (2–10 pS), insensitivity to voltage, cooperative activation by Ca^{2+} in the 0.3–0.7 μM range through tightly associated calmodulin, and voltage-dependent block by intracellular cations resulting in inward rectification [26]. These channels are subject to regulation by phosphorylation/dephosphorylation events [27], and heterotetramers can form. They are expressed in the nervous system (where they can regulate the firing pattern of neurons) and in various other organs, including heart, liver and muscle, and cell types – such as endothelial cells and lymphocytes – where they often share their functions with $K_{Ca}3.1$. Like the other members of the family, they can co-localize with specific Ca^{2+} sources in a cell type-specific manner.

$K_{Ca}3.1$ (SK4, Gardos channel, IK1, $IK_{Ca}1$, Kcnn4) is closely related to $K_{Ca}2$ and is not as ubiquitous as $K_{Ca}1.1$, but it is nonetheless widespread, being present in, e.g., erythrocytes, lymphocytes, liver and pancreas, but not in excitable cells. Thus, the presence of $K_{Ca}3.1$ in cardiomyocytes has not been reported, although the channel is expressed by vascular smooth muscle, endothelial and blood cells [22] and cardiac fibroblasts [28] and is therefore definitely present in the heart as a whole. It is highly expressed in a variety of cancer cell lines, including prostate PC-3 [29], breast MCF-7 [30] and glial GL-15 [31] lines, and it is thus considered a possible target for anti-cancer intervention [24]. Like the other Ca^{2+}-dependent K^+ channels, it functions in several physiological processes. It has been shown, for example, that $K_{Ca}2$ and $K_{Ca}3.1$ are important for agonist-evoked NO synthesis in vascular endothelial cells, vasorelaxation, and vascular smooth muscle cell (VSMC) proliferation and migration (and thus in atherosclerosis) [32–34]. $K_{Ca}3.1$ plays a major role in colonic salt secretion [35,36]. The channel has a non-ohmic single-channel conductance of 10–90 pS, is voltage-insensitive and responds to $[Ca^{2+}]$ in the sub-μM range, again due to a calmodulin molecule tightly associated to each monomeric unit.

The functions of the mitochondrial K^+ cycle have been identified as the control of matrix volume and the regulation of ROS production [2,3,37–39]. The former, a widely accepted notion, is in turn important for the regulation of metabolic processes and the optimisation of bioenergetic performance [40–42]. The latter, a more controversial issue [4,6,43–48], is considered to impact on cell-wide signalling and cell fate, and to explain the protection afforded by administration of K^+ channel openers against I/R damage, thought to involve activation of the RISK salvage pathway and downstream inhibition of the mitochondrial permeability transition [49–52]. Enhanced ROS generation appears to depend on the development of an inward K^+ current across the mitochondrial membrane, since it is also induced by the ionophore valinomycin [53,54] and by the viral protein p13, which causes K^+ influx [55]. In fact, similar effects are reportedly produced by activation of either mtK_{ATP} or $mtBK_{Ca}$, and Ca^{2+}-sensitive K^+ channels may constitute one of the links between mitochondrial Ca^{2+} handling and ROS generation [56].

Contrary to the cell protecting role assigned to mtK_{ATP} and $mtBK_{Ca}$, $mtK_V1.3$ promotes apoptosis [11]. As recapitulated in the paper by Gulbins et al. 2010 [12], in lymphocytes the pro-apoptotic BH3-only protein Bax, after incorporating into the OMM, interacts with the vestibule of IMM $K_V1.3$ channels, in a manner reminiscent of the mode of action of channel-blocking peptide toxins such as MgTx and ShK. This leads, again, to the production of ROS which in this case favour the detachment and release of cytochrome c. In principle, $mtK_{Ca}3.1$ may act either way: it is expected to open (before $K_{Ca}1.1$ because of its higher affinity for Ca^{2+}) as matrix Ca^{2+} increases, thus perhaps contributing to ROS production and eventually to MPTP inhibition, but an interaction of its periplasmic domain with Bax imbedded in the OMM was also a possibility, which we have verified.

2. Materials and methods

2.1. Materials, cells and mitochondria

Ionomycin and DC-EBIO (5,6-dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one) were from Tocris Cookson (Bristol, UK). Cyclosporin A was from LD Labs (Woburn, MA, USA), Annexin V-Fluos was from Roche (Milan), charybdotoxin was from Alomone Labs (Jerusalem, Israel). All other reagents and materials were from Sigma (Milan) unless specified.

Cells were grown in Dulbecco's modified Eagle medium (DMEM) plus 10 mM HEPES buffer, 10% (v/v) fetal calf serum (Invitrogen), 100 U/mL penicillin G, 0.1 mg/mL streptomycin, 2 mM glutamine (GIBCO) and 1% nonessential amino acids (100× solution; GIBCO), in a humidified atmosphere of 5% CO_2 at 37 °C. mtDsRed-transfected HeLa cells were obtained as already described for HCT116 cells [20]. Mitochondria for patch-clamp experiments were obtained by differential centrifugation [57]. For Western blotting a further purification was obtained by centrifugation (8500 *g*, 10 min, 4 °C) on a discontinuous Percoll gradient composed of 60, 30 and 18% Percoll in TES buffer (300 mM sucrose, 10 mM TES, 0.5 mM EGTA, pH 7.4). The floating material was discarded, and the fraction at the lower interface collected and washed three times by centrifugation at 17,000 *g* for 5 min. The final pellet was resuspended in TES buffer to form fraction 3 in Fig. 1.

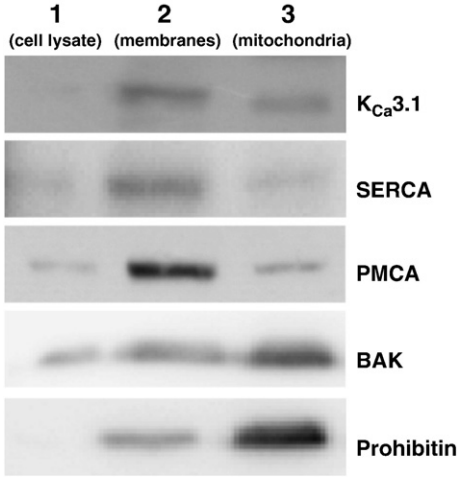

Fig. 1. $K_{Ca}3.1$ is not abundant in C-26 mitochondria. A representative experiment showing Western blots of the same preparations (1: cell lysate; 2: membrane-enriched fraction; 3: Percoll-purified mitochondria). The same amount of proteins (50 μg) was loaded in each lane. The blots on each horizontal line were obtained from the same PAGE gel and were blotted and developed together. The bands containing mitochondrial markers (29 kDa Bak, 30 kDa prohibitin) are more intense in the Percoll-purified fraction, while those corresponding to contamination markers (110 kDa SERCA, 140 kDa PMCA) and to $K_{Ca}3.1$ (55 kDa) decrease. Similar results were obtained in 2 other experiments.

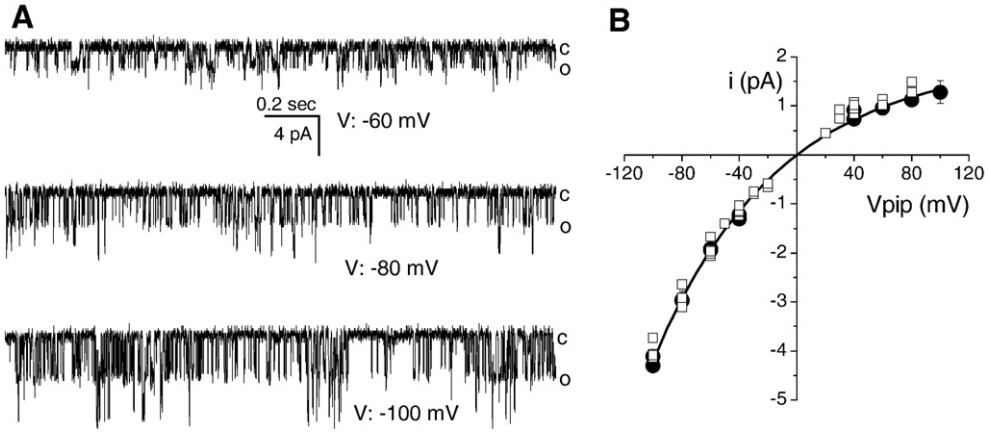

Fig. 2. mtK$_{Ca}$3.1 in HeLa mitochondria. A) Exemplary current records at the indicated voltages, in standard symmetrical 150 mM KCl medium. B) i–V plot. ●: averaged single-channel amplitude data ($N = 6$ to 13) from the experiment in A), fitted with an exponential. Error bars indicate standard deviations. □: analogous data from 5 separate experiments on HCT116 mitoplasts, under the same conditions (error bars have been omitted for clarity).

2.2. Western blotting

Samples, dissolved in sample buffer, allowed to stand for 15 min at R.T. and boiled for 5 min, were subjected to SDS-PAGE in 10% acrylamide minigels and transferred to a polyvinyldifluoride (Pall Corporation, Pensacola, FLA, USA) sheet. Primary antibodies used were: anti-Bak NT rabbit polyclonal (Upstate Biotechnology; cat. n. 06-536); anti-prohibitin mouse monoclonal (Lab Vision MS-261-P); anti-SERCA-2 ATPase mouse monoclonal (Affinity BioReagents MA3-910); anti-PMCA ATPase mouse monoclonal (Affinity BioReagents MA3-914); polyclonal anti-K$_{Ca}$3.1 (Alomone Labs APC-064) was raised in rabbit against a peptide comprising aa 350–363 of the rat channel. The immunogenic peptide was purchased from Alomone. Secondary antibodies (Calbiochem) were horseradish peroxidase-conjugated and used with chemiluminescence detection (Pierce) using film or digital imaging by a Bio-Rad ChemiDoc XRS apparatus.

2.3. Recombinant proteins

hBax(ΔC) (amino acids 1–170) and hBclxL were cloned into pGEX-3X as GST fusion proteins, expressed in BL21A1 and purified from bacterial lysates using glutathione-Sepharose [11,12].

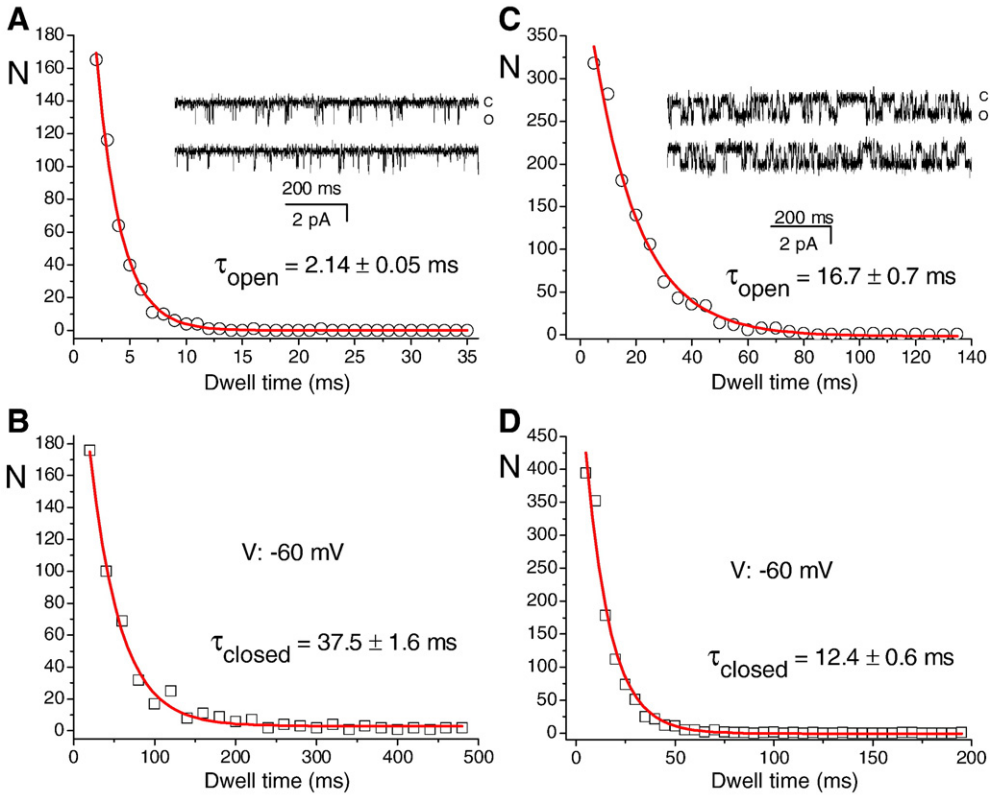

Fig. 3. mtK$_{Ca}$3.1 exhibits distinct kinetic behaviours. Distributions of channel open (panels A and C) and closed (B and D) times fitted with a single exponential. Panels A and B and C and D report data from two different experiments on HCT116 mitoplasts. Bin width: A: 1 ms; B: 20 ms; C and D: 5 ms. Insets in A) and C) show exemplary current records. The experiments were conducted in symmetrical 150 mM KCl medium, at $V = -60$ mV.

2.4. Vitality and apoptosis assays

For cell growth/viability tetrazolium reduction (MTT) assays, HCT116 cells were seeded in standard 96-well plates and allowed to grow in DMEM (200 µL) for 24 h. The growth medium was then replaced with medium that contained the desired compounds (from stock solutions in DMSO). The DMSO final concentration was ≤0.5% in all cases. Four wells were used for each condition. The solution was substituted by a fresh aliquot twice, at intervals of 24 h. At the end of the third 24 h period, the medium was removed, cells were washed with PBS, and 100 µL of PBS containing 10% CellTiter 96® AQ_{UEOUS} One solution (Promega) were added into each well. After 1 h of colour development at 37 °C, absorbance at 490 nm was measured using a Packard Spectra Count 96-well plate reader.

For the evaluation of apoptosis we plated HCT116 cells (300,000 cells/well) in a 6-well plate. After attachment overnight, cells were treated for 2, 6 and 12 h with the desired drugs (in the example of Fig. 6B: Tram 1 µM, Verapamil 30 µM (as inhibitor of MDR pumps), arachidonic acid 250 µM, as indicated) in DMEM or HBSS. After treatment, cells were harvested, washed with PBS, resuspended in FACS Buffer (Hepes 10 mM; NaCl 135 mM, $CaCl_2$ 5 mM, pH 7.4) and incubated with propidium iodide and Annexin V-Fluos in the dark at 37 °C for 15 min. Samples were then immediately analyzed by using a Beckton Dickinson FACScan flow cytometer (BD Biosciences). Data were processed using CellQuest® (BD Biosciences) and WinMDI2.8 (freeware; http://facs.scripps.edu/software.html, by Joe Trotter, the Scripps Institute) software. For DNA fragmentation assays, after washing the cells were resuspended in PBS, an equal volume of 70% ethanol was slowly added under continuous stirring, and the suspension was incubated overnight at 4 °C or for 30 min at R.T. After this permeabilization step, during which fragmented DNA is lost, the cells were washed in PBS, resuspended in PBS with RNAse A (20 µg/mL, 1400 units/mL) and incubated for 30 min at R.T. 5 µg/mL of propidium iodide were then added and the incubation continued for 20 min on ice in the dark, after which the samples were analyzed as above. Propidium fluorescence intensity is proportional to the amount of (unfragmented) DNA present.

2.5. Electrophysiology

Patch-clamp experiments on swollen mitochondria (mitoplasts) and perforated-patch whole-cell experiments were conducted as described in [20]. Axon pClamp software was used for voltage control and data analysis. For experiments with mitoplasts the voltages reported are those corresponding to the mitochondrial matrix. Inward currents are considered negative and plotted downwards. In the perforated-patch experiments illustrated in Fig. 5 all agents were bath-applied with a superfusion system (Rapid Solution Changer RSC-200; BioLogic Science Instruments, Claix, France). The time for solution exchange was about 500 ms; the solution was perfused via a glass pipette positioned at 300–500 µm from the cell. The maximal DMSO concentration in the recording solution was always less than 1%.

3. Results

A point of obvious relevance for an understanding of the physiological role of $mtK_{Ca}3.1$ is whether its localization in mitochondria is a peculiarity of the HCT116 cell line (human colorectal carcinoma), in which it was discovered, or is instead a common occurrence in cells expressing the channel. We therefore verified whether we could obtain evidence of its presence in mitochondria isolated from a few cell lines. Somewhat to our surprise, we could not reliably detect $mtK_{Ca}3.1$ in either of two colorectal adenocarcinoma cell lines: Caco-2, a human line (not shown), or C-26, a murine one (Fig. 1). In both cases the channel is present at the cell level, but in Western blots on progressively purer mitochondrial fractions the intensity of its band decreases as those of PM and ER markers likewise decrease and those of mitochondrial markers increase. Such a trend does not completely rule out the presence of the protein in mitochondria, but it does indicate that, if present at all, it has a low abundance.

$mtK_{Ca}3.1$ turned out instead to be present in HeLa, of human cervix adenocarcinoma origin (Fig. 2), and in mouse embryonic fibroblasts, non-cancerous cells (not shown). $K_{Ca}3.1$ is known to be expressed both by HeLa [58] and by fibroblasts [59]. In the experiment of Fig. 2, the recordings were obtained from a red-fluorescing mitoplast, isolated from HeLa cells expressing the mitochondria-targeted mtDsRed [20]. The data plotting the averaged single-channel current amplitudes vs. voltage (panel B, full circles) are shown to fall on the same curve as analogous data obtained from HCT116 mitochondria (squares), indicating that the same channel is involved. Our attempts to establish the presence of $mtK_{Ca}3.1$ in rat heart mitochondria have failed so far to provide a reliable answer.

Observation of the activity of $mtK_{Ca}3.1$ at the single-channel level allowed us to identify previously undescribed electrophysiological characteristics. The channel exhibits at least two well-defined rapid kinetic modes: a more frequently encountered one with a short mean open dwell time (time constant (τ) ~2 ms), a longer closed dwell time (τ ~40 ms) and a correspondingly low (voltage-independent) open probability (Fig. 3A and B), and one with longer-lasting (τ ~17 ms) openings and shorter closures (τ ~12 ms) (Fig. 3C and D). "Bursting" behaviour, conductance and voltage dependence of the unitary conductance and of the open probability were similar for the two forms; channels operating in either mode appeared to be equally sensitive to inhibition by TRAM-34 or clotrimazole, two specific inhibitors of intermediate-conductance Ca^{2+}-activated K^+ channels (not shown).

Rarely, the channel also operated in a lower-than-usual conductance state. In the example shown in Fig. 4, a channel with a chord conductance of about 22 pS at −70 mV gave way to the usual activity with a chord conductance of about 36 pS. Since the two types of activity were never observed together, and a subsequent addition of clotrimazole abolished all activity (not shown), we attribute both conductances to one $mtK_{Ca}3.1$ channel.

$mtK_V1.3$ interacts with the pro-apoptotic protein Bax, as shown, among other evidence, by patch-clamp experiments in which whole-cell current conducted by PM $K_V1.3$ was inhibited by externally applied recombinant GST-Bax(ΔC) [11]. We therefore checked whether a similar interaction might take place between $K_{Ca}3.1$ in the plasma membrane of HCT116 cells and this pro-apoptotic protein. Stable macroscopic K_{Ca} currents, recorded in the perforated-patch mode, were activated by applying the Ca^{2+} ionophore ionomycin (0.5 µM) plus the small/intermediate K_{Ca} channel opener DC-EBIO (100 µM). Time courses of the current in response to the various agents were built by plotting the outward current assessed at 0 mV from voltage ramps from −100 to 100 mV ($V_{holding}$ = 0 mV), applied every 5 s (Fig. 5). After stabilization of the current, GST-Bax(ΔC) was applied at 8.25 or 13.2 nM. This had no effect on the amplitude of the ionomycin + DC-EBIO-elicited current, which was instead fully blocked by the $K_{Ca}3.1$ channel antagonist TRAM-34 (3 µM) (N = 3). This observation indicates that there is no $K_{Ca}3.1$ block by GST-Bax(ΔC) at any voltage, and no modification of the permeation properties of the channel. Using the same experimental protocol, we also verified the effects of GST-BclxL (30 nM) on the $K_{Ca}3.1$ currents. GST-BclxL was, as expected, similarly ineffective (not shown).

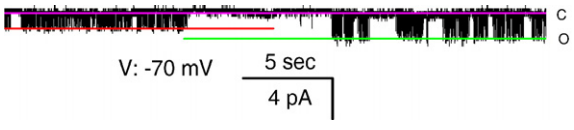

Fig. 4. $mtK_{Ca}3.1$ can adopt a lower-conductance state. A continuous current record showing the activity of a $mtK_{Ca}3.1$ channel in an HCT116 mitoplast. The red and green horizontal lines highlight the difference in current amplitude, the purple line indicates the background current level. See text for details.

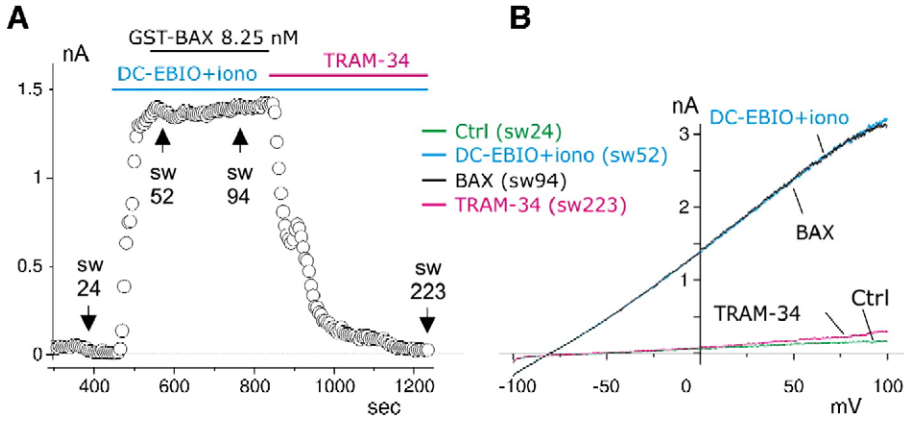

Fig. 5. Bax has no effect on $K_{Ca}3.1$ currents in HCT116 cells. A) Time course of the iono + DC-EBIO-activated $K_{Ca}3.1$ current from a HCT116 cell in whole-cell perforated configuration. Each data point represents the $K_{Ca}3.1$ current assessed at 0 mV from voltage ramp pulses delivered every 5 s under varying experimental conditions: i) control, ii) ionomycin + DC-EBIO (500 nM + 100 μM) alone, iii) iono + DC-EBIO (500 nM + 100 μM) plus GST-Bax(ΔC) (8.25 nM), and iv) ionomycin + DC-EBIO (500 nM + 100 μM) plus TRAM-34 (3 μM). B) Representative current traces in response to voltage ramps from − 100 to + 100 mV (1 s duration, $V_{holding}$: 0 mV), under the various pharmacological conditions shown in panel A. Numbers indicate the corresponding sweep in the time course display in panel A.

Using HCT116 cells, we verified whether the $K_{Ca}3.1$-specific, membrane-permeable inhibitor TRAM-34, by itself or in combination with inhibitors of MDR efflux pumps (Cyclosporin A, Probenecid, Verapamil, and Tamoxifen), had any effect on cell vitality or could induce apoptosis or enhance apoptosis induction by staurosporin. Assessment was carried out using cell counting, the MTT assay ($N = 3$), Annexin/propidium staining ($N = 8$) and DNA fragmentation assays ($N = 3$) (see Materials and methods for details), using staurosporin, indometacin and arachidonic acid as control apoptosis inducers. We could not detect an effect on either cell growth or cell death. Fig. 6 shows two representative experiments. The same result was obtained using the impermeable and less selective blocker charybdotoxin (not shown).

4. Discussion

The data presented above prove that the presence of the intermediate-conductance Ca^{2+}-activated K^+ channel is not a peculiarity of HCT116 cells (nor of cancerous cells, since it is present in the mitochondria of MEF). The mitochondrial subpopulation of $K_{Ca}3.1$ seems to be regulated case by case. Thus, in cell lines of colonic origin it may be detectable (HCT116) or undetectable (Caco-2, C-26). This in turn suggests (although it does not prove) that this population does not originate from an insufficiently selective targeting, i.e., $K_{Ca}3.1$ does not appear in mitochondria "by mistake", but presumably as a consequence of physiological events peculiar to each cell type. Other cases of cell type-specific localization of a protein in mitochondria as well as in another cellular compartment have been identified. For example, COX-2 is present in the mitochondria of several cancer cell lines, but not in those of fibroblasts or endothelial cells, whereas calcium-independent phospholipase A2 was found in the mitochondria of fibroblasts as well as in some cancerous cell lines [60].

One way a regulation of $K_{Ca}3.1$ localization might take place is via modulation of membrane/protein traffic, a signalling-controlled process (e.g.: [61,62]). One possibility is caveolae/lipid rafts-mediated traffic. A traffic route between caveolae, the endosomal system, the ER and Golgi has been identified [63–66], and a functional interaction of caveolae with mitochondria as well is suggested by proteomics profiling of the caveolae [67,68]. Caveolin-1 is targeted to various cellular compartments, including mitochondria, depending on cell type [69]. Garlid and coworkers have proposed that vesicular signalosomes originating from caveolae migrate to mitochondria, bind to OMM receptors and activate mtK_{ATP} via phosphorylations [70,71]. An interaction of the endosomal endocytosis pathway with mitochondria is also suggested by some observations [72]. Delivery of membrane proteins to mitochondria may also conceivably take place at ER-mitochondrion contact sites. These involve a differentiated ER membrane, MAM [73,74], are mediated by proteins such as PACS [75,76] and mitofusin 2 [77] and are sites of intermembrane transfer of phospholipids [78]. Regulation at the membrane-traffic level might result in the protein being present in mitochondria at different levels depending on cellular factors. Hypothetically, this might also explain the contradictory results in the literature on the presence or lack of K_{ATP} and $K_{Ca}1.1$ mitochondrial subpopulations.

IMM channels are furthermore subject to regulation at the molecular level. In the case of mtK_{ATP}, evidence has been presented for an

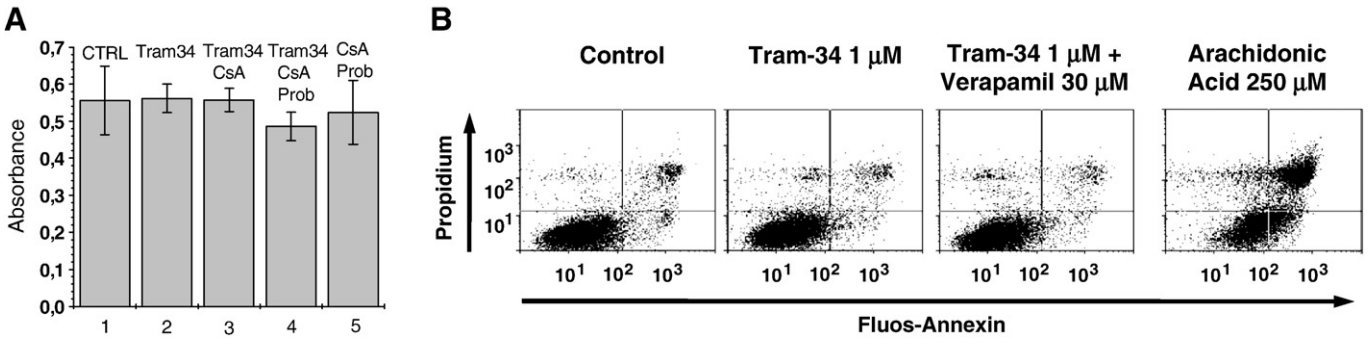

Fig. 6. TRAM-34 has no effect on HCT116 cell proliferation and does not induce cell death. A) Representative results of a tetrazolium salt (MTT) reduction assay. Cells were sowed and assayed after three days of growth (see Materials and methods for details). Conditions: 1: control (no addition); 2: TRAM-34, 2 μM; 3: TRAM-34, 2 μM, and Cyclosporin A, 2 μM; 4: TRAM-34, 2 μM, Cyclosporin A, 2 μM, and Probenecid, 100 μM; 5: Cyclosporin A, 2 μM, and Probenecid, 100 μM. B) A representative apoptosis assay using flow cytometry (FACS). Cells were incubated in DMEM in the presence of the specified substances for 6 h, then treated with propidium and Annexin as described in Materials and methods.

interaction with and activation by PKCε [79]. Interestingly, PKCε activation can cause caveolin-1- and cholesterol-dependent internalization of PM K_{ATP} channels in vascular smooth muscle cells [80]. PKG has also been proposed to be involved in signalling targeting mtK_{ATP} [81]. $mtK_{Ca}1.1$ is regulated by PKA [82–84]. Whether analogous processes impact on $mtK_{Ca}3.1$ is unknown, but it seems plausible to associate different kinetic and conductance properties (Figs. 3 and 4) with differences in post-translational modifications.

Roles have been assigned to $K_{Ca}3.1$ in a variety of cellular processes (see the Introduction and [20]), but its function can apparently be largely taken over by its smaller- and larger-conductance relatives, so that knock-out animals exhibit relatively mild phenotypes [85,86]. At the cell-culture level we also found it hard to identify clear short-term consequences of its inhibition at the plasma membrane (with charybdotoxin, not shown) or throughout the cell (with TRAM-34) (Fig. 6). In principle $mtK_{Ca}3.1$ ought to be able to mimic, at least to an extent, $mtK_{Ca}1.1$ functions, with the possibly relevant difference of activating at lower matrix Ca^{2+} levels (sub-μM vs. μM range). It is interesting in this context that the two channels have been recently found to interact, with $K_{Ca}3.1$ inhibiting $K_{Ca}1.1$ [87,88]. We had hypothesized [20] that $mtK_{Ca}3.1$ might act similarly to $mtK_V1.3$ [11] and interact with Bax. Experimental verification however has failed to support this hypothesis (Fig. 5). It seems therefore that the interaction of Bax with K^+ channels does not require simply a set of negative charges in the channel vestibule, but depends on more specific features. On the basis of computational simulations and of previous mutagenesis studies [89], Yu et al. [90] have identified 5 channel residues, Tyr-400, Asp-402, His-404, Asp-386 and Gly-380, as being of major relevance for $K_V1.3$-toxin interactions. His-404 is absent in $K_{Ca}3.1$ (Gly-380 corresponds to an Ala residue in $K_{Ca}3.1$). His-404 accounts for the pH-dependence of the interaction between the channel and some toxins, as well as with Bax [12]. Aspartates at positions 375 and 376 in the turret region of Kv1.3 do not have an equivalent in $K_{Ca}3.1$ [89], so that the formal negative charge in this region, presumably important for the interaction with Bax Lys128, is 1/monomer in $K_{Ca}3.1$ vs. 3 in $K_V1.3$.

In summary, the roles of $mtK_{Ca}3.1$ in mitochondrial and cellular physiology may be at least in part redundant, in analogy to what seems to be the case at the cellular level, since fully functional mitochondria exist without it (in cells not expressing the channel but apparently also in cells which do express it). The possibility remains that the channel may play a major role in some specific, yet to be discovered, process, but the data presented here do not support a key role in the control of cell proliferation or drug-induced apoptosis. Where present, $mtK_{Ca}3.1$ is fully expected to contribute to the IMM K^+ conductance following an increase in matrix $[Ca^{2+}]$; the relevance of this contribution in comparison with other K^+ permeation pathways remains to be explored. The mechanisms accounting for its presence in mitochondria need to be investigated, and may well apply to other proteins with multiple localisations.

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