Studying mitochondrial Ca\(^{2+}\) uptake – A revisit

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**Abstract**

Mitochondrial Ca\(^{2+}\) sequestration is a well-known process that is involved in various physiological and pathological mechanisms. Using isolated suspended mitochondria one unique mitochondrial Ca\(^{2+}\) uniporter was considered to account ubiquitously for the transfer of Ca\(^{2+}\) into these organelles. However, by applying alternative techniques for measuring mitochondrial Ca\(^{2+}\) uptake evidences for molecularly distinct mitochondrial Ca\(^{2+}\) carriers accumulated recently. Herein we compared different methodological approaches of studying mitochondrial Ca\(^{2+}\) uptake. Patch clamp technique on mitoplasts from endothelial and HeLa cells revealed the existence of three and two mitoplast Ca\(^{2+}\) currents (I\(_{\text{AMito}}\)), respectively. According to their conductance, these channels were named small (s-), intermediate (i-), large (l-) and extra-large (xl-) mitoplast Ca\(^{2+}\) currents (MCC). i-MCC was found in mitoplasts of both cell types whereas s-MCC and l-MCC or xl-MCC were exclusively found in mitoplasts from endothelial cells or HeLa cells. The comparison of mitochondrial Ca\(^{2+}\) signals, measured either indirectly by sensing extra-mitochondrial Ca\(^{2+}\) or directly by recording changes of the matrix Ca\(^{2+}\) showed different Ca\(^{2+}\) sensitivities of the distinct mitochondrial Ca\(^{2+}\) uptake routes. Subpopulations of mitochondria with different Ca\(^{2+}\) uptake capacities in intact endothelial cells could be identified using Rhod-2/AM. In contrast, cells expressing mitochondrial targeted pericam or cameleon (4mDiDcppv) showed homogeneous mitochondrial Ca\(^{2+}\) signals in response to cell stimulation. The comparison of different experimental approaches and protocols using isolated organelles, permeabilized and intact cells, pointed to cell-type specific and versatile pathways for mitochondrial Ca\(^{2+}\) uptake. Moreover, this work highlights the necessity of the utilization of multiple technical approaches to study the complexity of mitochondrial Ca\(^{2+}\) homeostasis.

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1. Introduction

Mitochondria achieve a multitude of biochemical functions (Graier et al., 2007; McBride et al., 2006) of which the combustion of substrates coupled to the transfer of electrons to molecular oxygen, proton pumping across the inner mitochondrial membrane yielding ATP generation are the best-known examples (Kennedy and Lehninger, 1949). Because of such central role in energy metabolism mitochondria are often referred to as the cell’s powerhouses.

The ability of mitochondria to rapidly transform their morphological appearance is an additional remarkable feature of these organelles (Chen, 1988; Koshiba et al., 2004; Liu et al., 2009). Mostly, mitochondria create worm-like structures, which are constantly remodeled by fission, fusion and branching (Bereiter-Hahn and Jendrach, 2010). Moreover, mitochondria are transiently tethered to other cell structures such like the endoplasmic reticulum (ER) (Csordas et al., 1999; de Brito and Scorrano, 2008; Merkworth and Langer, 2008), the nucleus (Liu and Butow, 2006), other organelles (Stemberger et al., 1984), the plasma membrane (Malli et al., 2003), the cytoskeleton (Ball and Singer, 1982), and linked to motor-proteins for directed movements (Liu and Hajnoczky, 2009).

Another striking feature of mitochondria is their ability to sequester calcium ions (Ca\(^{2+}\)), nature’s most widely used second messenger (Berridge et al., 2000; Dhalla, 1969; Graier et al., 2007; Malli and Graier, 2010). Mitochondrial Ca\(^{2+}\) uptake plays an important role in the cell’s physiological and pathological signal transduction (Berridge et al., 2003; Demaurex and Distelhorst, 2003; Duchen et al., 2008). The transfer of Ca\(^{2+}\) into mitochondria is assumed to impact cell signaling basically by two processes. Firstly, mitochondrial Ca\(^{2+}\) uptake shapes the amplitude, the temporal- and spatial pattern of local as well as global extra-mitochondrial Ca\(^{2+}\) signals, which considerably impacts on Ca\(^{2+}\)-sensitive processes upon cell stimulation (Knot et al., 2005) (i.e. Ca\(^{2+}\) buffer function). Secondly, elevated mitochondrial Ca\(^{2+}\) is crucially important for cellular processes such as respiration and ATP production (Wiederkehr et al., 2011), autophagy (Decuypere et al., 2011),...
protein folding (Osibow et al., 2006), gene expression (Cao and Chen, 2009) and, upon excessive Ca\textsuperscript{2+} overload, the initiation of programmed cell death (apoptosis) (Giorgi et al., 2008).

The phenomenon of mitochondrial Ca\textsuperscript{2+} uptake has been discovered in the early 1960s (Deluca and Engstrom, 1961) when it was recognized that isolated mitochondria have a high capacity to sequester Ca\textsuperscript{2+}. In these experiments mitochondrial Ca\textsuperscript{2+} uptake was recognized indirectly by measuring the reduction of the extra-mitochondrial Ca\textsuperscript{2+} concentration upon repetitive applications of Ca\textsuperscript{2+} portions to isolated, suspended, respiring mitochondria. With such kinds of experiments the enormous capacity of mitochondria to absorb Ca\textsuperscript{2+} was discovered and mitochondrial Ca\textsuperscript{2+} uptake was well characterized as the so-called mitochondrial Ca\textsuperscript{2+} uniport (MCU) (reviewed by Malli and Graier, 2010). It was shown that the MCU is indeed a Ca\textsuperscript{2+} ion channel (Kirichok et al., 2004). More recently, one component of the elusive MCU has been discovered by integrative genomics (Baughman et al., 2011; De Stefani et al., 2011). Remarkably, the MCU of isolated mitochondria exhibited a rather low Ca\textsuperscript{2+} affinity (Gunter et al., 1994). Based on this low Ca\textsuperscript{2+} affinity, mitochondrial Ca\textsuperscript{2+} uptake was considered as physiologically irrelevant, and mitochondria were thought to work as passive Ca\textsuperscript{2+} sinks (reviewed by Santo-Domingo and Demaurex, 2010). However, due to the development of mitochondria targeted luminescent or later on fluorescent protein-based Ca\textsuperscript{2+} sensors that allowed a direct measurement of mitochondrial Ca\textsuperscript{2+} signals, mitochondria were demonstrated to actively contribute to the cells Ca\textsuperscript{2+} homeostasis (Rizzuto et al., 1992; Jiang et al., 2009; Perocchi et al., 2010; Trenker et al., 2008; Miyawaki et al., 1999, 2003; Nagai et al., 2002; Szabadkai et al., 2004). Direct measurements of mitochondrial Ca\textsuperscript{2+} signals in cells using this novel technique revealed distinct modes of mitochondrial Ca\textsuperscript{2+} uptake with high Ca\textsuperscript{2+} sensitivities not seen in isolated mitochondria before (Waldeck-Weiermair et al., 2010a,b). In line with this work, the contribution of two proteins to distinct mitochondrial Ca\textsuperscript{2+} uptake routes in one given cell was described (Waldeck-Weiermair et al., 2011).

In this study different techniques ranging from indirect measurements using isolated, suspended mitochondria to direct recordings of mitochondrial free Ca\textsuperscript{2+} concentration in intact living cells were compared. This comparison highlights the crucial differences of the various techniques and calls for caution for a direct comparison of results obtained by the various methods. In addition, this work provides evidence for molecularly distinct, probably interrelated, pathways for mitochondrial Ca\textsuperscript{2+} sequestration.

2. Materials and methods

2.1. Cell culture

Human umbilical vein endothelial cells (EA.hy926) (Edgeell et al., 1983) (passage number > 80) were grown on Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% HAT (5 mM hypoxanthin, 20 μM aminopterin, 0.8 mM thymidine), 50 μM penicillin and 50 μg/ml streptomycin (PAA Laboratories, Pasching Austria) at 37 °C, 5% CO\textsubscript{2}. HeLa cells were grown on DMEM containing 10% FCS, 50 μU/ml penicillin and 50 μg/ml streptomycin. The rat pancreatic beta cells (INS-1; 832/13) was cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM glutamine, 1 mM Na-pyruvate (PAA Laboratories, Pasching Austria). HeLa cells were grown on DMEM containing 10% FCS, 50 μU/ml penicillin and 50 μg/ml streptomycin. The rat pancreatic cell line (INS-1; 832/13) was cultured in RPMI 1640 medium containing 10% FCS, 10 mM HEPES, 2 mM glutamine, 1 mM Na-pyruvate (PAA Laboratories, Pasching Austria) at 37 °C, 5% CO\textsubscript{2}.

2.2. Isolation of mitochondria and mitoplasts

Mitochondria were freshly isolated by differential centrifugation from wild type yeast (Daum et al., 1982) or liver tissue of mice (Storrie and Madden, 1990) as previously described (Trenker et al., 2008). Isolated mitochondria were suspended in storage buffer composed of 10 mM Hepes, 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM succinate, 2 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM DTT, pH adjusted to 7.4 with KOH (Lactan, Graz, Austria).

Mitoplasts were prepared from isolated mitochondria of HeLa and an endothelial cell line (EA.hy926) cells by differential centrifugation steps using both methods of mitochondria isolation kit for cultured cells (Thermo Scientific 89874, USA) and an organelle isolation protocol described by Frezza et al. (2007). Mitoplast formation was achieved by incubation of isolated mitochondria in 4 volumes hypotonic solution (5 mM Hepes, 5 mM sucrose, 1 mM EGTA, pH adjusted to 7.4 with KOH < 10 mM) and equilibrated on ice with 1 volume hypotonic solution (750 mM KCl, 80 mM Hepes, 1 mM EGTA, pH adjusted to 7.4 with KOH < 10 mM) after 15 min.

2.3. Mitoplast patch clamp recordings

All measurements were performed in mitoplast-attached configuration of the patch-clamp technique at room temperature. Gigaohm seals were established on the membrane section opposite to the cap region. Patch pipettes were pulled from glass capillaries using a Narishige puller (Narishige Co., Ltd., Tokyo, Japan), fire-polished and had a resistance of 8–12 MΩ when filled with a solution containing 150 mM CaCl\textsubscript{2}, 10 mM Hepes, or low chloride solution with 55 mM Ca-methanesulfonate, 50 mM CaCl\textsubscript{2}, 10 mM Hepes, pH adjusted to 7.2 with Ca(OH). Bath solution contained 150 mM KCl, 1 mM EGTA, 1 mM EDTA, 10 mM Hepes, pH adjusted to 7.2 with KOH. 10 μM Cyclosporin A (Tocris Bioscience, Bristol, UK) and 20 μM CGP-37157 (Ascent Scientific Ltd., Bristol, UK) were added to both the bath and pipette solution. Ruthenium red (RuR) (10 μM) (Merck Chemicals Ltd., Darmstadt, Germany) was added when indicated. Currents were recorded using a patch-clamp amplifier (EPC7, List Electronics, Darmstadt, Germany) at a bandwidth of 3 kHz. Data collection was performed using Clampex software of pClamp (V9.0, Axon Instruments). Signals obtained were low pass filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices), and digitized with a sample rate of 10 kHz using a Digidata 1200A A/D converter (Axon Instruments, Foster City, CA, USA).

2.4. Indirect measurement of mitochondrial Ca\textsuperscript{2+} uptake of isolated mitochondria and permeabilized cells

Indirect measurement of free mitochondrial Ca\textsuperscript{2+} uptake was performed with Calcium-Green \textsuperscript{5} N. Samples of isolated mitochondria (0.25 mg/ml) and HeLa cells (2.5 × 10\textsuperscript{6} cells/ml, harvested by trypsinization) were suspended in high potassium buffer composed of 110 mM KCl, 500 mM K\textsubscript{2}HPO\textsubscript{4}, 1 mM MgCl\textsubscript{2}, 20 mM Hepes, 10 μM EGTA, 5 mM succinate, pH adjusted to 7.3 with KOH. In isolated mitochondria rotenone was supplied to 4 μM, while digitonin (Sigma, Vienna, Austria) was added to University of Graz, Austria). For single-cell analysis cells were grown on glass coverslips, and transiently transfected with the FRET-based mitochondrial sensor 4mtD3cpv using Transfast (Promega, Mannheim, Germany) according to the manufacturer’s protocol. Alternatively, EA.hy926 cells were stably transfected with mitochondrial targeted ratiometric pericam (RPmt).
30 µM to permeabilize cells. Calcium-Green® 5 N indicator was added to both samples to a final concentration of 0.25 µM. Ca²⁺ uptake of permeabilized cells and isolated mitochondria in suspension was measured on a fluorescence spectrophotometer (Hitachi F-4500; Hitachi, Inula, Vienna, Austria) at 506 nm for excitation and 532 nm for emission.

2.6. Confocal imaging of intact cells and mitochondrial preparations

Z-scans were performed on a Nipkow-disk-based array confocal laser-scanning microscope, as described before (Trenker et al., 2008). Intact cells, on glass cover slips, were loaded with 5 µM Rhod-2/AM for 30 min at room temperature, excited at 514 nm (150 mW Ar laser, Laser Physics, USA) and fluorescence was monitored at 570 nm (E570LPv2, Chroma Technology Corp., Rockingham, VT, USA). Single-cell measurements of mitochondrial Ca²⁺ was done in endothelial cells either transfected with RPmt (Nagai et al., 2002) or 4MTd3cpv (Palmer et al., 2006) using a Zeiss Axiovert 200 M (Zeiss, Vienna, Austria) at 340 and 380 nm excitation (340HT15, 380HT15, Omega Optical, Brattleborough, VT, USA) and 510 nm emission filter (510WB40, Omega Optical), as described before (Trenker et al., 2008). Intact cells, on glass cover slips, were loaded with 5 µM Rhod-2/AM at 30 min for room temperature, excited at 514 nm (150 mW Ar laser, Laser Physics, USA) and fluorescence was monitored at 570 nm (E570LPv2, Chroma Technology Corp., Rockingham, VT, USA). Single-cell measurements of mitochondrial Ca²⁺ was done in endothelial cells either transfected with RPmt (Nagai et al., 2002) or 4MTd3cpv (Palmer et al., 2006) using a Zeiss Axiovert 200 M (40× oil objective, Zeiss), a polychromator illumination system (VisiChrome High Speed, Xenon lamp, Visitron Systems, Puchheim, Germany) and a thermoelectric-cooled CCD camera (Photometrics Coolsnap HQ, Visitron Systems) or a Nikon Eclipse TE300 (Plan Fluor 40× oil objective, Nikon, Japan), a polychromator lamp (Opti Quip 770, USA) and a liquid-cooled CCD camera (Photometrics Quantix KAF, Roper Scientific, Tucson, AZ, USA). Data acquisition and analysis was done using the MetaMorph or VisiView software (Visitron Systems). Glass cover slips were mounted into an experimental chamber equipped with a perfusion system at a rate around 2 ml/min. Cells were put into resting solution prior to experiments composed of 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 2.6 mM NaHCO₃, 440 µM KH₂PO₄, 340 µM Na₂HPO₄, 10 mM d-glucose, 0.1% vitamins, 0.2% essential amino acids, 1% penicillin/streptomycin, pH adjusted to 7.4 with NaOH. For experiments cells were perfused with Ca²⁺-free solution, composed of 138 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM d-glucose, 1 mM EGTA, pH adjusted to 7.4 with NaOH. Cells were stimulated by the addition of either 100 µM carbachol, 100 µM ATP or 100 µM histamine and 15 µM BHQ, RPmt was used to simultaneously measure free mitochondrial Ca²⁺ at 410/438 nm excitation and changes in pH at 485 nm excitation, both with emission at 535 nm (433DF15/535AF26, Omega Optical). Excitation of the 4MTd3cpv was applied at 440 ± 10 nm (440AF21, Omega Optical), and emission was recorded at 480 and 535 nm using a beam splitter (Optical Insights, Visitron Systems). Excitation filters were adjusted through a filter-wheel (MAC 6000/5000, Ludl Electronic Products, Hawthorne, NY, USA). Devices were controlled and data was recorded by MetaFluor 4.6r3 software or VisiView 2.0.3 (Universal Imaging, Visitron Systems).

2.5. Direct measurement of free mitochondrial Ca²⁺ by chemical fluorophores and mitochondrial targeted biosensors

Single isolated mitochondria were incubated with Fura-2/AM (3.3 µg/ml) in the dark for 1 h at RT. Samples of 20 µl suspended Fura-2/AM-loaded isolated mitochondria were incubated for 8 min on a coverslip until they got attached. Samples were perfused with high potassium buffer for measurement on a Zeiss Axiovert 200 M (Zeiss, Vienna, Austria) at 340 and 380 nm excitation (340HT15, 380HT15, Omega Optical, Brattleborough, VT, USA) and 510 nm emission filter (510WB40, Omega Optical), as described before (Trenker et al., 2008). Intact cells, on glass cover slips, were loaded with 5 µM Rhod-2/AM at 30 min for room temperature, excited at 514 nm (150 mW Ar laser, Laser Physics, USA) and fluorescence was monitored at 570 nm (E570LPv2, Chroma Technology Corp., Rockingham, VT, USA). Single-cell measurements of mitochondrial Ca²⁺ was done in endothelial cells either transfected with RPmt (Nagai et al., 2002) or 4MTd3cpv (Palmer et al., 2006) using a Zeiss Axiovert 200 M (40× oil objective, Zeiss), a polychromator illumination system (VisiChrome High Speed, Xenon lamp, Visitron Systems, Puchheim, Germany) and a thermoelectric-cooled CCD camera (Photometrics Coolsnap HQ, Visitron Systems) or a Nikon Eclipse TE300 (Plan Fluor 40× oil objective, Nikon, Japan), a polychromator lamp (Opti Quip 770, USA) and a liquid-cooled CCD camera (Photometrics Quantix KAF, Roper Scientific, Tucson, AZ, USA). Data acquisition and analysis was done using the MetaMorph or VisiView software (Visitron Systems). Glass cover slips were mounted into an experimental chamber equipped with a perfusion system at a rate around 2 ml/min. Cells were put into resting solution prior to experiments composed of 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM Hepes, 2.6 mM NaHCO₃, 440 µM KH₂PO₄, 340 µM Na₂HPO₄, 10 mM d-glucose, 0.1% vitamins, 0.2% essential amino acids, 1% penicillin/streptomycin, pH adjusted to 7.4 with NaOH. For experiments cells were perfused with Ca²⁺-free solution, composed of 138 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM d-glucose, 1 mM EGTA, pH adjusted to 7.4 with NaOH. Cells were stimulated by the addition of either 100 µM carbachol, 100 µM ATP or 100 µM histamine and 15 µM BHQ, RPmt was used to simultaneously measure free mitochondrial Ca²⁺ at 410/438 nm excitation and changes in pH at 485 nm excitation, both with emission at 535 nm (433DF15/535AF26, Omega Optical). Excitation of the 4MTd3cpv was applied at 440 ± 10 nm (440AF21, Omega Optical), and emission was recorded at 480 and 535 nm using a beam splitter (Optical Insights, Visitron Systems). Excitation filters were adjusted through a filter-wheel (MAC 6000/5000, Ludl Electronic Products, Hawthorne, NY, USA). Devices were controlled and data was recorded by MetaFluor 4.6r3 software or VisiView 2.0.3 (Universal Imaging, Visitron Systems).

3. Results

3.1. Isolated mitoplasts exhibit multiple distinct Ca²⁺ currents that vary depending on the cell type chosen for isolation

Using isolated mitochondria is the most invasive and elaborated method to investigate the phenomenon of mitochondrial Ca²⁺ uptake. However, this technique is certainly a very useful and insightful approach to look at mitochondrial signaling. One powerful feature of using isolated organelles is the possibility to apply the patch clamp technique, which allows the characterization of intracellular Ca²⁺ channels even on the single channel level. We isolated mitochondria from different cell lines and tissues, stained them with MitoTracker®, and imaged them on a fluorescence microscope. Isolated mitochondria always appeared as small spherical structures with diameters ranging from 0.25 to 1.5 µm (Trenker et al., 2008) that tend to aggregate independently from the source used (Fig. 1A). In order to patch the inner mitochondrial membrane (IMM), mitochondria were swelled in hypotonic media, which leads to the rupture of the outer mitochondrial membrane (OMM) obtaining larger objects, the so-called mitoplasts. Mitoplasts from HeLa and endothelial cells were sometimes large in size and frequently contained remnants of the OMM, visible next to the mitotracker-stained particle (Fig. 1B). Non-fluorescent particles are equally attributed to OMM remnants as to disrupted mitochondria, which do not accumulate mitotracker molecules. Mitoplasts were
used to measure Ca$^{2+}$ currents in the mitoplast-attached configuration. Patch experiments were carried out in buffers containing cyclosporin A for inhibition of mitochondrial permeability transition pore opening and CGP-37157 for blocking mitochondrial Na$^{+}$/Ca$^{2+}$ exchanger (Cox and Matlib, 1993; Cox et al., 1993) and Letm1 (Jiang et al., 2009). Moreover, experiments were carried out with low chloride buffer in the pipette, to test whether or not obtained currents are, at least in part, carried by Cl$^-$ outward currents. There were no differences in the observed current conductance and density whether low chloride or high chloride concentration was present in the pipette (data not shown), thus, confirming that the currents measured are carried by Ca$^{2+}$ movements. Experiments with a high Ca$^{2+}$ concentration in the patch pipette revealed strong inward currents at negative potentials (Fig. 1C, black trace), pointing to Ca$^{2+}$ uniporter activities in these mitoplasts. All Ca$^{2+}$ currents ($I_{\text{CaMito}}$) of mitoplasts from HeLa as well as endothelial cells could be blocked by 10 μM RuR in the pipette (Fig. 1C, red trace). Notably, the amplitudes of $I_{\text{CaMito}}$ measured were not stable over time. Intermittently different $I_{\text{CaMito}}$ responses to voltage ramps in one given mitoplast could be observed. This finding might either point to fluctuations of the activity of one given MCU channel or to the co-existence of more than one distinct current amplitude of single channel events in mitoplasts from endothelial cells (Fig. 1D, left panel) and HeLa cells (Fig. 1D, right panel).
panel). Time-lapsed recordings were taken at holding potentials of either −100, −140, −150 or −160 mV and revealed clear single channel openings in mitoplasts of both cell types. Out of these recordings three and two distinct conductances of \( \text{ICAMito} \) could be distinguished in endothelial and HeLa cells, respectively (Table 1). These \( \text{ICAMito} \) were subsequently named as small (s-MCC), intermediate (i-MCC) and extra large (xl-MCC) mitoplast/mitochondrial Ca\(^{2+}\) current (Table 1). Additional biophysical parameters like the current’s appearance, mean open time, mean closed time and open probabilities (nPo) are given in Table 1. The i-MCC was found in mitoplasts from both cell types. In contrast, s-MCC that required high negative voltages and i-MCC were only found in endothelial mitoplasts while xl-MCC was exclusively found in HeLa cell mitoplasts (Table 1).

3.2. Isolated mitochondria exhibit different Ca\(^{2+}\) uptake pathways

A less laborious approach to study mitochondrial Ca\(^{2+}\) uptake of isolated mitochondria is to measure the reduction of applied bath Ca\(^{2+}\) boluses to suspended mitochondria using a fluorescence Ca\(^{2+}\) indicator in the medium. We used Calcium-Green\(^{5}\) N for this purpose studying Ca\(^{2+}\) uptake of isolated mitochondria from mice liver. Thereby, striking differences in the clearance of added Ca\(^{2+}\) could be observed among different experimental approaches, although using isolated mitochondria from the same batch and origin under the same experimental conditions (Fig. 1E). Basically, this finding is in line with the fluctuating activities of mitochondrial Ca\(^{2+}\) channels observed in mitoplasts, but might rather point to variances in the stability of the quality of isolated mitochondria from mice liver. Moreover, mitochondrial Ca\(^{2+}\) signals, if measured directly by loading Fura-2/AM in isolated organelles, revealed quite homogeneous signals of single isolated mitochondria using fluorescence microscopy (Fig. 1F).

3.3. Mitochondrial Ca\(^{2+}\) uptake measured in permeabilized cells unveil high and low Ca\(^{2+}\) sensitive pathways

Similar to the signals observed with isolated mitochondria the indirect measurement of mitochondrial Ca\(^{2+}\) uptake of digitonin-permeabilized HeLa cells using Calcium-Green\(^{5}\) N showed a fast decline in the free extra-mitochondrial Ca\(^{2+}\) concentration upon additions of Ca\(^{2+}\) portions (Fig. 2). The kinetics of mitochondrial Ca\(^{2+}\) uptake in permeabilized cells remained unaltered for several repeats of Ca\(^{2+}\) boluses over a considerable period of time, pointing to the high capacity of mitochondria to absorb Ca\(^{2+}\) under these conditions. However, after a certain number of cumulative additions of Ca\(^{2+}\) to a suspension of permeabilized HeLa cells, the Ca\(^{2+}\) concentration of the medium strongly increased, indicating mitochondrial Ca\(^{2+}\) overload and opening of a mitochondrial permeability transition pore (Huang et al., 2000; Hunter and Haworth, 1979). The number of the Ca\(^{2+}\) pulses that induced mitochondrial Ca\(^{2+}\) overload and permeability transition pore opening naturally correlated with the cell number used (Fig. 2A (1.3 × 10\(^{6}\) cells) and B (5.9 × 10\(^{6}\) cells)).

In line with early results using isolated mitochondria and the Calcium-Green\(^{5}\) N method (Eberhard and Erne, 1991) the minimal Ca\(^{2+}\) concentration capable of activating mitochondrial Ca\(^{2+}\) uptake of suspended permeabilized cells was assessed to be explicitly higher than 3 \( \mu \text{M} \) (Fig. 2C). We hypothesized that the low Ca\(^{2+}\) affinity of the mitochondrial Ca\(^{2+}\) uptake pathway of permeabilized HeLa cells recognized, was overestimated due to the Ca\(^{2+}\) buffer capacity of Calcium-Green\(^{5}\) N in the medium, which naturally lowers the free Ca\(^{2+}\) concentration available on sites of mitochondrial Ca\(^{2+}\) uptake. In order to test this assumption, analogous experiments were performed using the high Ca\(^{2+}\) sensitive Fura-2 instead of Calcium-Green\(^{5}\) N to measure Ca\(^{2+}\) in the medium. Using Fura-2 in the medium of the suspension, however, confirmed the low Ca\(^{2+}\) affinity of mitochondrial Ca\(^{2+}\) uptake of permeabilized HeLa cells (Fig. 2D). Similar data were obtained using permeabilized endothelial cells (data not shown).

For comparison, similar experiments were performed on the single cell level with permeabilized endothelial cells that stably expressed the fluorescence sensor protein mitochondrial targeted pericam (RPmt). In contrast to the experiments above, this approach highlighted clear rises of \([\text{Ca}^{2+}]_{\text{mito}}\) in permeabilized cells, even at a Ca\(^{2+}\) concentration lower than 1 \( \mu \text{M} \) (Fig. 2E). Moreover, this experimental approach revealed that the kinetics of mitochondrial Ca\(^{2+}\) uptake in one given model (i.e. permeabilized cells) crucially depends on the sensor type and method chosen. Notably, using the rather high-sensitive intraluminal Ca\(^{2+}\) sensor pericam already at concentrations of 1 \( \mu \text{M} \) bath Ca\(^{2+}\) a strong mitochondrial Ca\(^{2+}\) sequestration was detected, while the sensor signal got saturated at concentrations \( > 1 \mu \text{m \text{M}} \) bath Ca\(^{2+}\). In contrast, using a Ca\(^{2+}\) sensor in the bath (i.e. Calcium-Green\(^{5}\) N or Fura-2) bath Ca\(^{2+}\) concentrations \( > 3 \mu \text{M} \) are essential for measuring a significant mitochondrial Ca\(^{2+}\) uptake (Fig. 2F).

Similar to its inhibitory effect on all \( \text{ICAMito} \) (Fig. 1C), Ruthenium-Red (RuR) inhibited Ca\(^{2+}\) uptake in isolated mitochondria and permeabilized cells (Fig. 3). RuR mostly shows inability of passing cellular membranes. Consequently, the usability of RuR in intact cells is limited. The potent uncoupling agent carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone (FCCP) inhibited Ca\(^{2+}\) uptake in intact cells and mitochondrial preparations (Fig. 3). Notably, because RuR and FCCP do not impact other Ca\(^{2+}\) handling organelles, the inhibition of Ca\(^{2+}\) uptake by RuR and FCCP reflects a decreased mitochondrial Ca\(^{2+}\) activity. Nevertheless, the impact of RuR and FCCP on the rates of mitochondrial Ca\(^{2+}\) uptake differed within the various model/method used. While almost no Ca\(^{2+}\) uptake was detectable in presence of both chemical agents in permeabilized cells (Fig. 3C and D), in isolated suspended mitochondria FCCP was more efficient than RuR (Fig. 3B and D). In case of single isolated mitochondria both compounds appeared to be less active in terms of their inhibitory potential on mitochondrial Ca\(^{2+}\) uptake (Fig. 3B and D).

Table 1

| Density | Conductance (pS) | Mean open time (ms) | Mean closed time (ms) | nPo |
|---------|----------------|--------------------|----------------------|-----|
| EC      |                |                    |                      |     |
| s-MCC   | 4 out of 14    | 7.69 ± 1.42        | 2.44 ± 0.51          | 19.20 ± 15.88 | 0.88 ± 1.27 |
| i-MCC   | 9 out of 14    | 13.37 ± 2.44       | 3.14 ± 0.58          | 11.26 ± 3.82 | 1.11 ± 0.64 |
| l-MCC   | 3 out of 14    | 34.52 ± 4.05       | 4.57 ± 5.40          | 52.07 ± 35.87 | 0.70 ± 0.96 |
| HeLa    |                |                    |                      |     |
| i-MCC   | 15 out of 22   | 14.30 ± 2.67       | 3.6 ± 4.65           | 13.68 ± 9.62 | 0.67 ± 0.62 |
| xl-MCC  | 9 out of 22    | 74.33 ± 2.57       | 1.9 ± 0.88           | 6.08 ± 1.50 | 1.11 ± 0.66 |

Abbreviations: number of patches (n), small (s), intermediate (i), large (l) and extra large (xl) mitochondrial Ca\(^{2+}\) current (MCC) in endothelial cells and HeLa cells; open-probability of all channel events (nPo).

Data presented as mean ± standard deviation.
Fig. 2. Variable responses of Ca\textsuperscript{2+} indicators in permeabilized cells. Digitonin-treated HeLa cells were exposed to exogenously added 50 μM Ca\textsuperscript{2+} pulses repeated after 100–200 s and mitochondrial Ca\textsuperscript{2+} uptake was measured with Calcium-Green\textsuperscript{5} N in the bath. Cells show similar rates in uptake, but number of repeats varies in relation to cell quantity, 1.3 × 10\textsuperscript{6} cells (A, n = 3) and 5.9 × 10\textsuperscript{6} cells (B, n = 3). (C) Representative tracings of mitochondrial Ca\textsuperscript{2+} uptake in suspended permeabilized cells in response to various Ca\textsuperscript{2+} concentrations in Calcium-Green\textsuperscript{5} containing buffer (n = 3). (D) Representative tracings of mitochondrial Ca\textsuperscript{2+} uptake in suspended permeabilized cells in response to various Ca\textsuperscript{2+} concentrations in Fura-2 containing buffer (n = 3). (E) Representative tracings of mitochondrial Ca\textsuperscript{2+} uptake in suspended permeabilized cells stably expressing RPmt in response to various Ca\textsuperscript{2+} concentrations. Data was normalized to % max of 1 \text{F}_{438}/F_{0}\text{ at } 1 \text{μM free Ca}\textsuperscript{2+} concentration (Waldeck-Weiermair et al., 2010a) and shown as mean ± SEM (n = 8–17). (F) Statistical evaluation of the kinetics of mitochondrial Ca\textsuperscript{2+} sequestration presented as % max. slope of mitochondrial [Ca\textsuperscript{2+}] signal of each method upon various Ca\textsuperscript{2+} concentrations in permeabilized cells stably expressing RPmt (left panel; n = 8–17) or Calcium-Green\textsuperscript{5} N in the bath (right panel; n = 3–5), presented as mean ± SEM.
3.4. Rhod-2 staining reveals distinct subpopulations of mitochondria with different basal Ca$^{2+}$ levels and Ca$^{2+}$ uptake capacity within intact endothelial cells

Rhod-2 is a red fluorescent Ca$^{2+}$ indicator of low molecular weight that is frequently used to study mitochondrial Ca$^{2+}$ signals (Fonteriz et al., 2010). We loaded endothelial cells with the acetoxymethyl ester of Rhod-2 (Rhod-2/AM) in order to test the suitability of this method. A detectable and consistent staining of all mitochondria with Rhod-2 was observed only if cells were treated for at least 30 min with 1 μM Rhod-2/AM at room temperature (Fig. 4A, left panel). Exposure of these distinctly loaded cells to 514 nm laser light remarkably reduced the selectivity of mitochondrial staining of Rhod-2 within few minutes (Fig. 4A, right panel). In order to reduce such putative phototoxicity, cells were moderately loaded with Rhod-2/AM. Reduction of both the loading time (10 min) and the concentration of Rhod-2/AM (300 nM) yielded a spotty and apparent incomplete staining of mitochondria within one given cell (Fig. 4B, left panel). Notably, most of the mitochondria did not become apparent on the fluorescence microscope using the moderate loading procedure. However, upon stimulation with the IP$_3$-generating agonist histamine caused a significant increase in fluorescence (Fig. 4B, right panel), indicating sufficient Rhod-2 loading to respond to Ca$^{2+}$ rises in almost all mitochondria. The comparison of Rhod-2 signals of mitochondria that exhibited a clear staining already prior to cell stimulation with those which were initially invisible, revealed distinct differences in their capability to respond to cellular Ca$^{2+}$ signals (Fig. 4C and D). In one given cell, mitochondria that initially showed a high Rhod-2 signal under resting conditions, only moderately responded to stimulation with histamine (Fig. 4C and D, green trace). In contrast, the greater subpopulation of mitochondria with very low basal Rhod-2 signals, strongly responded to the IP$_3$-dependent Ca$^{2+}$ mobilization (Fig. 4C and D, red trace). These findings point to the existence of distinct subpopulations of mitochondria with different basal Ca$^{2+}$ levels and capacities to absorb Ca$^{2+}$. Moreover, the finding that mitochondria with high basal Ca$^{2+}$ levels were almost incompetent to respond to cell stimulation, might point to a negative feedback of Ca$^{2+}$ on the mitochondrial Ca$^{2+}$ uptake pathway.

3.5. Mitochondrial Ca$^{2+}$ and H$^+$ signals measured with the genetically encoded sensors pericam and cameleon point to FCCP-sensitive mitochondrial Ca$^{2+}$ uptake machineries in intact cells

Mitochondrial targeted ratiometric pericam (RPmt) is a circular permuted fluorescent protein (FP) that was developed by Miyawaki and colleagues in 2001 (Nagai et al., 2001) and exhibits an exceptional targeting efficiency into mitochondria (Fig. 5A) based on a N-terminal targeting sequence of 15 amino acids from the mitochondrial cytochrome C oxidase subunit IV (COX IV). This protein-based Ca$^{2+}$ sensor principally consists of a permuted yellow fluorescent protein that is flanked by calmodulin and the Ca$^{2+}$-calmodulin binding domain M13 (Fig. 5B). Pericam absorbs blue light showing two excitation maxima, particularly in the range of 410–440 nm and 480–490 nm, respectively, while emitting green light at a maximum of approximately 535 nm (Fig. 5C). Ca$^{2+}$ binding to RPmt in intact cells mainly affected the fluorescence of this sensor when excited with 410–440 nm. In contrast, the less Ca$^{2+}$ sensitive fluorescence of pericam at an excitation of 480–490 nm was highly sensitive to changes in pH (Fig. 5C). These properties of pericam offer the possibility to measure changes in Ca$^+$ and H$^+$ simultaneously (Fonteriz et al., 2010; Waldeck-Weiermair et al., 2011).

We used an endothelial cell line stably expressing RPmt in order to study the impact of the chemical uncoupler FCCP on the mitochondrial Ca$^{2+}$ and H$^+$ homeostasis of intact cells (Fig. 5D and E). Cell stimulation with the IP$_3$-generating agonist histamine triggered a fast and transient increase of mitochondrial Ca$^{2+}$ levels.
(Fig. 5D, upper panel), which was subsequently associated with a significant acidification of the mitochondrial matrix (Fig. 5D, lower panel). Addition of FCCP during cell stimulation promptly reduced $[\text{Ca}^{2+}]_{\text{mito}}$ (Fig. 5D, upper panel) and naturally yielded a

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**Fig. 4.** Different levels of Rhod-2 loading unmask mitochondrial subpopulations. Endothelial cells were treated for 30 min with 1 µM Rhod-2/AM at room temperature (A, left image) and exposed for 2 min at 514 nm (A, right image). Moderately Rhod-2/AM-loaded cells (i.e., 300 nM for 10 min) under resting conditions (B, left panel) and after stimulation with histamine (B, right panel). (C and D) Subpopulations of mitochondria from one cell are shown that exhibited either low basal Rhod-2 signals (green) or high basal Rhod-2 staining (red) prior to cell stimulation. The comparison of those two mitochondrial subpopulations revealed distinct differences in their capability to respond to IP3-dependent Ca$^{2+}$ mobilization (C). Corresponding regions of interest are marked with green and red numbers. Timestamps are given in the right bottom corner in min:sec (D).
pronounced increase of the mitochondrial H+ concentration (Fig. 5D, lower panel). Removal of FCCP was without any effect on [Ca2+]mito (Fig. 5D, upper panel), but led to a slow recovery of mitochondrial H+ levels (Fig. 5D, lower panel). In line with these findings, pretreatment of cells with FCCP strongly inhibited mitochondrial Ca2+ signals in intact cells (Fig. 5E).

Cameleons are ingenious Ca2+ sensors that consist of two different fluorescent proteins, mostly the cyan fluorescent protein (CFP) and the yellow fluorescent protein (YFP), which have overlapping spectral properties (Miyawaki et al., 1997). Ca2+ levels in living cells expressing cameleons can be visualized as Ca2+ binding to cameleons rapidly changes the conformation of the sensor increasing Förster resonance energy transfer (FRET) from CFP to YFP (Fig. 6A). Cameleons are thus ratiometric Ca2+ sensors as the Ca2+ induced increase in FRET is naturally associated with a parallel decrease of the CFP fluorescence. Since the introduction of the first cameleon in 1997, several improved derivates of this Ca2+ sensor with proper Ca2+ sensitivities, higher FRET-efficiencies and increased pH stabilities have been developed (McCombs and Palmer, 2008; Miyawaki et al., 1999). However, probably due to the rela-
tive bulkiness of cameleons, these Ca\textsuperscript{2+} sensors exhibited low targeting specificity. This characteristic could be significantly improved by the introduction of a tandemly duplicated mitochondrial targeting sequence of COX VIII (4mtD3cpv) (Filippin et al., 2005; Palmer et al., 2006). In our experiments, approximately 20\% of the endothelial cells expressing 4mtD3cpv exhibited a clear mitochondrial staining of the Ca\textsuperscript{2+} sensor without any mistargeting to the cytosol after 24 h (Fig. 6B, upper panel) and exhibited perfect mirror-like signaling of the donor and the acceptor fluorescence upon cell stimulation (Fig. 6C). Notably, cells with partially mistargeted 4mtD3cpv had often fragmented organelles (Fig. 6B, middle panel) while in cells with high levels of mistargeted cameleon in the cytosol mitochondria appeared highly fragmented (Fig. 6B, lower panel). Overall, these findings may indicate that the expression of 4mtD3cpv potentially impact the morphology of mitochondria. Thus, considering the possibility that mitochondrial Ca\textsuperscript{2+} handling and the morphology of these organelles are interrelated phenomena, the use of this sensor and the interpretation of respective signals should be done with caution.

In order to compare the pH sensitivity of 4mtD3cpv with that of RPmt analogous experiments were performed using FCCP (Figs. 5E vs. 6D). In cells without (Fig. 6D, continuous line) and with mistargeted sensor (Fig. 6D, dotted line), addition of FCCP had only little effects on the fluorescence properties of 4mtD3cpv, pointing to the pH stability of this Ca\textsuperscript{2+} sensor. Mitochondrial Ca\textsuperscript{2+} signals in response to Ca\textsuperscript{2+} mobilization upon histamine and BHQ were clearly inhibited by the chemical uncoupler (Fig. 6D and E). However, in cells with mistargeted sensor, cytosolic Ca\textsuperscript{2+} signals could be measured in parallel (Fig. 6E), confirming the finding that FCCP predominantly impacts the mitochondrial Ca\textsuperscript{2+} homeostasis in this particular cell type.

The usability of 4mtD3cpv for studying mitochondrial Ca\textsuperscript{2+} signals was further tested by imaging IP\textsubscript{3}-dependent changes of [Ca\textsuperscript{2+}]\textsubscript{mito} in various cell types (Fig. 6F). The amount of cells expressing 4mtD3cpv successfully targeted to the mitochondria was approximately 30\% in HeLa, 70\% in OP-9 cells, 85\% in HL-1, and 65\% in INS-1 cells. Stimulation of HeLa cells with the IP\textsubscript{3}-generating agonist histamine and ATP in the absence of extracellular Ca\textsuperscript{2+} induced a fast and transient increase of [Ca\textsuperscript{2+}]\textsubscript{mito} in all cells measured, whereas some cells showed an oscillatory mitochondrial Ca\textsuperscript{2+} signal under these conditions (Fig. 6F, left upper panel). Ca\textsuperscript{2+} readdition to prestimulated HeLa cells elevated [Ca\textsuperscript{2+}]\textsubscript{mito} only in 2 out of 11 cells (Fig. 6F, left upper panel), despite the fact that [Ca\textsuperscript{2+}]\textsubscript{cyto} was always significantly enhanced in this experimental protocol (data not shown). Both the non excitable mouse stromal cell line OP-9 (Fig. 6F, right upper panel) and the HL-1 mouse cardiomyocytes (Fig. 6F, left lower panel) responded to ATP by a fast increase of [Ca\textsuperscript{2+}]\textsubscript{mito}, which was of higher amplitude in case of the OP-9 cells. Notably, some of the mouse cardiomyocytes showed basal oscillations of [Ca\textsuperscript{2+}]\textsubscript{mito} probably reflecting the generation of spontaneous action potentials within these excitable

![Fig. 6. Close to RTmt but less specific in targeting while essentially ratiometric: mitochondria-targeted cameleon for monitoring mitochondrial Ca\textsuperscript{2+} uptake. (A) Model with systematic structure of mitochondria-targeted cameleons. (B) Targeting of the cameleon 4mtD3cpv to mitochondria after 24 h of transient transfection in endothelial cells revealed few successful targeting to mitochondria (upper panels), cytosolic mistargeting to some degree (middle panel) and high level of mistargeting (lower panels). (C) Original tracings of basal FRET (F\textsubscript{535}) and the related CFP (F\textsubscript{480}) fluorescence in endothelial cells transiently expressing 4mtD3cpv. As indicated, cells were stimulated with 100 \mu M ATP. For normalization the respective F\textsubscript{0} curves (F\textsubscript{535} and F\textsubscript{480}) were extrapolated from existing basal values using GraphPad Prism 5. (D) Impact of 2 \mu M FCCP on basal mitochondrial Ca\textsuperscript{2+} levels monitored using perfectly targeted (continuous line) and mistargeted (dotted line) 4mtD3cpv. As indicated 2 mM Ca\textsuperscript{2+}, 100 \mu M histamine and 15 \mu M BHQ were added to maximally stimulate mitochondrial Ca\textsuperscript{2+} challenge under these conditions (n = 11–13). (E) Individual fluorescence of 4mtD3cpv that correspond to the experiments shown in (D). (F) Original tracings of the effect of IP\textsubscript{3}-dependent changes of [Ca\textsuperscript{2+}]\textsubscript{mito} due to histamine, ATP or carbachol (CCh) in several cell types that were transiently transected with 4mtD3cpv. Mean represented by bold black line with circles, single responses HeLa n = 11, OP-9 n = 10, HL-1 n = 10, INS-1 n = 8.]

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cells (Fig. 6F, left lower panel). Oscillations of \([\text{Ca}^{2+}]_{\text{mito}}\) with a smaller amplitude could be also observed in the β-cell line (INS-1) that showed a fast increase of mitochondrial \([\text{Ca}^{2+}]\) levels upon cell stimulation with carbachol (CCh) (Fig. 6F, right lower panel).

4. Discussion

Mitochondria are able to decode cytosolic \([\text{Ca}^{2+}]\) signals by sequestering these ions and the subsequent activation of \([\text{Ca}^{2+}]\)-dependent processes that, in turn, are crucial for the cell responsiveness and functions (Duchen and Szabadkai, 2010; Graier et al., 2007). Accordingly, mitochondrial \([\text{Ca}^{2+}]\) uptake is considered as an important cellular process that is relevant for both physiological and pathological cell signaling (Duchen et al., 2008).

Events of mitochondrial \([\text{Ca}^{2+}]\) uptake can be studied on isolated organelles with high temporal resolution using the patch camp technique. In addition fluorescent \([\text{Ca}^{2+}]\) sensors that either indirectly indicate the decline of extra-mitochondrial \([\text{Ca}^{2+}]\) upon mitochondrial \([\text{Ca}^{2+}]\) sequestration or directly measure mitochondrial \([\text{Ca}^{2+}]\) signals of the matrix of isolated mitochondria, mitochondria in permeabilized or intact cells are frequently used to study mitochondrial \([\text{Ca}^{2+}]\) signaling. Each method represents a distinct possibility of studying mitochondrial \([\text{Ca}^{2+}]\) uptake whereupon the most appropriate application of one protocol is down to the actual question to be investigated. Our comparison of different experimental approaches and protocols revealed that depending on the techniques used, different properties of mitochondrial \([\text{Ca}^{2+}]\) uptake are unmasked. Notably, caution is necessary in the interpretation of data elaborated with only one technique as discrepancies within data obtained with different techniques might be due to the distinct methodical approaches. Such discrepancies have recently led to controversies regarding the putative function of uncoupling proteins 2 and 3 (UCP2/3) as key components of mitochondrial \([\text{Ca}^{2+}]\) channels (Brookes et al., 2008; De Marchi et al., 2011; Trenker et al., 2008).

The choice of the technique used to study mitochondrial \([\text{Ca}^{2+}]\) uptake appears to be crucial and the decision might base on several considerations: In general, all approaches using isolated mitochondria offer the opportunity to be accessible for cell-impermeable substrates, and are adequate for proteomic studies. Moreover, it offers experiments using the patch clamp technique and, thus, the direct investigation of mitochondrial ion channels. Permeabilization methods require lower sample amount, summarize subsets of mitochondrial populations and do not directly change adjacent cell structure, preserving possible interactions with other organelles (Saks et al., 1998). Still, permeabilization may impede some of the intact cells properties and limits possibilities to studying signal transduction mechanisms due to a loss of cytoplasm. Finally, the undisturbed systemic view on the level of intact cells appears to be the most attractive, if one intends to investigate the complexity of mitochondrial functions in their natural environment and their participation in cellular signal transduction.

4.1. Patching mitoplasts

In this study we show evidence for the existence of two currents of different amplitudes that occurred alternately in a stochastic manner, thus, possibly pointing to different mitochondrial \([\text{Ca}^{2+}]\) channels in mitoplasts prepared from HeLa and endothelial cells.
To our knowledge this is the first time that mitochondrial Ca\textsuperscript{2+} channels have been characterized using the patch clamp technique in this particular cell lines that are frequently used to investigate mitochondrial signaling. Our finding of different mitochondrial Ca\textsuperscript{2+} channels in HeLa and endothelial cells is in line with a recent report that showed distinct mitochondrial Ca\textsuperscript{2+} channels of mitoplasts from human cardiac myocytes (Michels et al., 2009). However, because in the mitoplast-attached configuration used (i.e. high K\textsuperscript{+} in the medium) the actual potential of the mitoplast may not entirely be constant, changes of single channel amplitudes may reflect spontaneous alterations of the membrane potential of mitoplasts. Indeed such fluctuations in the membrane potential of the mitoplast might be responsible for the small shifts of the distinct current amplitudes in experiments with a presumably constant holding potential. The different conductances of currents found in our experiments may not necessarily prove the existence of multiple individual channels but different modes of one single channel for mitochondrial Ca\textsuperscript{2+} uptake (Spat et al., 2008; Szanda et al., 2008, 2010). Nevertheless, as individual currents also occur superimposed with rather distinct biophysical characteristics, the distinct ranges of current amplitudes/conductances obtained in mitoplasts of HeLa and endothelial cells, most likely reflect the co-existence of at least two separate inward Ca\textsuperscript{2+} currents. Evidently, these findings support the assumption of the co-existence of multiple and maybe cell type- and species specific mitochondrial Ca\textsuperscript{2+} channels (Michels et al., 2009). Interestingly, with its conductance of 13–14 pS, f-MCC that was found to exist in mitoplasts from endothelial and HeLa cells, is strikingly similar to the mCa1 found in non-failing cardiac myocytes (Michels et al., 2009), though the gating parameters were slightly different within the two studies. Moreover, the conductance (7 pS) and gating characteristics of endothelial s-MCC, described herein, meets that of the mCa2 in non-failing heart (Michels et al., 2009) in view of the close developmental association of endothelial cells with cardiac myocytes, their similarities in regard to the two mitochondrial Ca\textsuperscript{2+} inward currents might not be surprising and may further point to tissue specificity of the mitochondrial Ca\textsuperscript{2+} uptake machinery.

4.2. Different modes of mitochondrial Ca\textsuperscript{2+} uptake in permeabilized cells

Major differences in Ca\textsuperscript{2+} sensitivity of mitochondrial Ca\textsuperscript{2+} uptake were observed between indirect assessments and direct recordings of mitochondrial Ca\textsuperscript{2+} signals using permeabilized cells. Notably, while in experiments with permeabilized cells expressing RPmt a mitochondrial Ca\textsuperscript{2+} uptake at Ca\textsuperscript{2+} concentrations below 200 mM was measured, this highly Ca\textsuperscript{2+} sensitive uptake pathway/mode could not be observed when mitochondrial Ca\textsuperscript{2+} sequestration was indirectly measured in suspended permeabilized cells using a Ca\textsuperscript{2+} dye in the bath (i.e. “Calcium-Green 5 N technique”). The actual reason for this difference is not known. However, it has to be considered that the signals obtained, when measuring mitochondrial Ca\textsuperscript{2+} uptake of a population of permeabilized cells indirectly (i.e. “Calcium-Green 5 N technique”), might reflect the summary of multiple complex Ca\textsuperscript{2+} shuttling events involving the opening of the mitochondrial permeability transition pore as well. This assumption is supported by a recent report demonstrating maximal Ca\textsuperscript{2+} uptake of suspended mitochondria to depend on the mode of Ca\textsuperscript{2+} addition (Chalmers and Nicholls, 2003).

4.3. Mitochondrial Ca\textsuperscript{2+} signals of intact cells

Using FP-based Ca\textsuperscript{2+} sensors targeted to the mitochondria represents the most elegant way to study mitochondrial Ca\textsuperscript{2+} uptake in intact cells (Demaurex, 2005). Very recently, we used this method to assess different modes of mitochondrial Ca\textsuperscript{2+} uptake in endothelial cells (Waldeck-Weiermair et al., 2011). Thereby, the specific contribution of different proteins, that were shown to play important roles in mitochondrial Ca\textsuperscript{2+} uptake, was investigated. In regard to the contribution of uncoupling protein 2 and 3 (UCP2/3) experiments using siRNA mediated knock-down (Trenker et al., 2007; Waldeck-Weiermair et al., 2010a), expression of mutated proteins (Waldeck-Weiermair et al., 2010b), and overexpression of UCP 2/3 (Trenker et al., 2007; Waldeck-Weiermair et al., 2010a) revealed that these proteins fundamentally contribute to mitochondrial uptake of high and low Ca\textsuperscript{2+} signals in intact cells. Notably, under physiological low expression levels of UCP 2/3, these proteins exclusively contributed to mitochondrial Ca\textsuperscript{2+} uptake at sites of ER Ca\textsuperscript{2+} release (Waldeck-Weiermair et al., 2010a,b). In contrast, the leucine zipper EF hand-containing transmembrane protein 1 (Letm1), that was recently identified as a mitochondrial Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter (Jiang et al., 2009), entirely accomplished the transfer of entering Ca\textsuperscript{2+} into mitochondria in a UCP 2/3-independent, high Ca\textsuperscript{2+}-sensitive manner (Waldeck-Weiermair et al., 2011). Moreover, we used this method to directly assess the impact of mitochondrial calcium uptake 1 (MICU1), a protein that triggers mitochondrial Ca\textsuperscript{2+} uptake in HeLa cells (Perocchi et al., 2010), on mitochondrial Ca\textsuperscript{2+} signaling in intact endothelial cells. Hence, because siRNA-mediated knock-down (verified on mRNA level) of MICU1 failed to impact mitochondrial Ca\textsuperscript{2+} uptake in the endothelial cell the involvement of MICU1 in mitochondrial Ca\textsuperscript{2+} uptake in this particular cell type can be excluded (Waldeck-Weiermair et al., 2011). The contribution of the ryanodine receptor type 1 (Beutner et al., 2005; Ryu et al., 2010, 2011) and the very recently described mitochondrial Ca\textsuperscript{2+} uniporter protein (Baughman et al., 2011; De Stefani et al., 2011) to mitochondrial Ca\textsuperscript{2+} uptake in endothelial cells awaits investigation. In particular, the question remains, whether or not these proteins contribute to one given conductance/channel/Ca\textsuperscript{2+} entry pathway or achieve distinct Ca\textsuperscript{2+} entry routes into the mitochondria, like it has been recently described for UCP2/3 and Letm1 (Waldeck-Weiermair et al., 2011). Overall, these studies that were mainly based on direct measurements of mitochondrial Ca\textsuperscript{2+} uptake of intact cells using FP-based Ca\textsuperscript{2+} sensors (RPmt and 4mtD3cpv), indicate the co-existence of at least two molecularly distinct mitochondrial Ca\textsuperscript{2+} uptake pathways in one given cell type. These pathways might be necessary in order to properly integrate cytosolic Ca\textsuperscript{2+} signals into mitochondrial responses.

4.4. Conclusion

Here we demonstrate that different experimental approaches yield different views of mitochondrial Ca\textsuperscript{2+} uptake. There is increasing evidence that several different proteins accomplish the transfer of Ca\textsuperscript{2+} across the inner mitochondrial membrane during cell stimulation (Hajnoczy and Csoros, 2010; Malli and Graier, 2010) that may account for either a low Ca\textsuperscript{2+}-sensitive but high capacity or a high Ca\textsuperscript{2+}-sensitive but low capacity mitochondrial Ca\textsuperscript{2+} uptake pathway. Importantly, these different mitochondrial Ca\textsuperscript{2+} uptake routes/modes often become evident or remain undetectable depending on the protocols and techniques used. The scenario gains complexity if one considers multiple ways and mechanisms that may modulate the function of the proteins contributing to mitochondrial Ca\textsuperscript{2+} signaling in intact cells (Koncz et al., 2009; Szanda et al., 2010). Accordingly, mitochondrial Ca\textsuperscript{2+} uptake is still an enigmatic molecular process and to investigate this versatile and complex phenomenon the utilization of multiple techniques and methodical approaches appears necessary.

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