Ca\textsuperscript{2+} uptake in mitochondria occurs via the reverse action of the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger in metabolically inhibited MDCK cells

Ilse Smets, Adrian Caplanusi,* Sandra Despa,* Zsolt Molnar, Mihai Radu, Martin van de Ven, Marcel Ameloot, and Paul Steels

Department of Physiology, Limburgs Universitair Centrum/Transnationale Universiteit Limburg, Biomedisch Onderzoeksinstituut, B-3590 Diepenbeek, Belgium

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O VERLOAD IS an important determinant of ischemic injury, because an excessive increase in cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) might activate different Ca\textsuperscript{2+}-dependent catabolic enzymes such as phospholipases, proteases, and endonucleases. Therefore, excessive elevation of resting [Ca\textsuperscript{2+}]\textsubscript{i} is deleterious to almost all cell types and can be associated with either necrotic or apoptotic cell death (19, 45, 71). The toxic nature of Ca\textsuperscript{2+} requires any cytosolic Ca\textsuperscript{2+} load, including the Ca\textsuperscript{2+} loads seen in the normal regulation of a wide variety of physiological events, to be cleared quickly. Excessive cytosolic Ca\textsuperscript{2+} is removed from the healthy cell across the plasma membrane either by Ca\textsuperscript{2+}-ATPases or Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers (NCE). Ca\textsuperscript{2+} can also be sequestered into intracellular organelles such as the endoplasmic/sarcoplasmic reticulum (ER/SR) stores via Ca\textsuperscript{2+}-ATPases. Moreover, clearance of cytosolic Ca\textsuperscript{2+} loads by mitochondria has been demonstrated in a wide variety of cell types (6, 17, 27, 28, 61). Increases in mitochondrial Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) are believed to increase the production of ATP (27, 46, 61).

Mitochondria take up Ca\textsuperscript{2+} primarily through a uniporter system (4, 28, 61). The influx of Ca\textsuperscript{2+} into the matrix by this route is dependent on the electrochemical gradient for Ca\textsuperscript{2+}. This gradient is developed and maintained by the mitochondrial membrane potential (\(\Delta\Psi\textsubscript{m}\)), generally estimated to be on the order of 150–200 mV negative to the cytosol and by a low resting [Ca\textsuperscript{2+}]\textsubscript{m}, maintained primarily by the mitochondrial NCE. The second mode of inward Ca\textsuperscript{2+} transport is referred to as the “rapid mode” or RaM, because it is at least 300 times more rapid than uptake via the uniporter under the same conditions (27, 68). This RaM transports Ca\textsuperscript{2+} only for a brief period (a fraction of a second) during the initial phase of the cytosolic Ca\textsuperscript{2+} pulse. The driving force for Ca\textsuperscript{2+} uptake via the RaM is the Ca\textsuperscript{2+} electrochemical gradient, as it is for the uniporter. Consequently, the collapse of \(\Delta\Psi\textsubscript{m}\) in response to pathological conditions will limit mitochondrial Ca\textsuperscript{2+} uptake and may contribute to cellular pathophysiology (17, 61). Moreover, ischemia-induced cellular ATP depletion will hamper the other previously mentioned ATPases responsible for cytosolic Ca\textsuperscript{2+} clearance.

In this study, we examined the alterations in cellular and mitochondrial Ca\textsuperscript{2+} homeostasis during metabolic inhibition (MI) in Madin-Darby canine kidney (MDCK) cells, a cell line of distal tubular origin that exhibits many similarities to mammalian cortical collecting tubular cells (74). Inhibition of cellular metabolism was used as an experimental model to simulate ischemic cell injury. It was realized by inhibiting both cellular glycolysis [with 2-deoxyglucose (2-DG)] and oxidative phosphorylation [with cyanide (CN)]. The aim of the present study was to investigate whether ATP-depleted MDCK cells were able to clear the cytosolic Ca\textsuperscript{2+} overload during MI and to determine the underlying Ca\textsuperscript{2+}-transporting mechanism.

* Present addresses of coauthors: A. Caplanusi, Dept. of Medical Biochemistry, Carol Davila University of Medicine and Pharmacy, R-050474 Bucharest, Romania; S. Despa, Dept. of Physiology, Loyola University Chicago, Maywood, IL 60153; Z. Molnar, Dept. of Medical Chemistry, University of Szeged, H-6701 Szeged, Hungary; and M. Radu, Dept. of Health and Environmental Physics, Horia Hulubei National Institute for Physics and Nuclear Engineering, R-76900 Bucharest, Romania.

Address for reprint requests and other correspondence: I. Smets, MBW-Dept. of Physiology, Limburgs Universitair Centrum/Transnationale Universiteit Limburg, Biomedisch Onderzoeksinstituut, Universitaire Campus Gebouw D, B-3590 Diepenbeek, Belgium (E-mail: ilse.smets@luc.ac.be).

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This report shows that MI induces a transient increase in [Ca\(^{2+}\)] in MDCK cells. Our results suggest that the second-phase clearance of [Ca\(^{2+}\)], during MI is not due to either Ca\(^{2+}\) extrusion out of the cells nor Ca\(^{2+}\) storage into the ER but rather to Ca\(^{2+}\) uptake into the mitochondrial matrix. Furthermore, our findings strongly suggest that the mitochondrial Ca\(^{2+}\)-importing mechanism is not the unipor or the RaM but rather the mitochondrial NCE acting in the reverse mode.

**MATERIALS AND METHODS**

**Cell Culture**

MDCK cells (low passage number, 22–30) were kindly donated by Dr. H. De Smedt (Laboratory of Physiology, Leuven, Belgium). Cells were cultured in a 1:1 mixture of DMEM and Ham’s F-12, supplemented with 10% fetal calf serum, 14 mM L-glutamine, 25 mM NaHCO\(_3\), 100 U/ml penicillin, and 100 \(\mu\)g/ml streptomycin. Cells were maintained in a humidified 5% CO\(_2\) atmosphere at 37°C. The medium was renewed every 3–4 days. When cultured on permeable supports, the cells formed confluent monolayers with a resistance of 400–500 \(\Omega\)cm\(^2\). For all experiments, 1–2 \(\times\) 10\(^5\) cells were seeded onto round glass coverslips with a diameter of 24 mm. After 3–6 days of culture, confluent monolayers were used.

**Cellular ATP Content**

Confluent monolayers of MDCK cells were washed with normal saline solution and incubated with a solution containing metabolic inhibitors (see below for details) for various time intervals ranging from 5 to 60 min. Control cells were incubated in normal saline solution. ATP measurements were performed with a luciferin-luciferase-based assay kit (Molecular Probes, Eugene, OR). The reaction buffer contained 150 \(\mu\)g/ml luciferin, 1.25 \(\mu\)g/ml luciferase, 5 mM MgSO\(_4\), 1 mM dithiothreitol, 25 mM Tricine, 0.1 mM EDTA, and 0.1 mM azide, pH 7.8. The cells were solubilized in 450 \(\mu\)l of somatic cell ATP-releasing agent (Sigma, St. Louis, MO) for 30 s. Fifty microliters of cell extract was added to 450 \(\mu\)l of reaction buffer. ATP levels were measured with a luminometer (model 1250, Wallac, Turku, Finland). Calibration was performed with several standard ATP solutions in the concentration range 10 \(-8\)–10 \(-5\) M. The results are expressed as percent change compared with control.

**Fluorescence Imaging Microscopy**

The coverslips with MDCK cells were mounted into a homebuilt holder and placed on the stage of an inverted epifluorescence microscope (Zeiss Axiovert 100, Jena, Germany). A thermostatic heating chamber (set at 37°C) enclosed the microscope stage. After measurement of the background signal, the cells were loaded with an appropriate fluorescent indicator. Both before and after dye loading, cells were gently washed several times with normal saline solution. Fluorescence was elicited by illumination with an XBO 75 W/2 OFR xenon lamp (Osram, Berlin-Munich, Germany). Excitation filters were inserted into a computer-controlled shuttered filter wheel (Lambda 10–2, Sutter Instrument, Novato, CA), which allows fast alternation between different excitation filters. All optical filters and dichroic mirrors were obtained from Chroma Technology (Brattleboro, VT). The excitation light was directed to the sample by a dichroic mirror and a Zeiss LD Achromat objective (×40/0.6 corr.). Fluorescence collected by the objective was transmitted through the dichroic mirror and a band-pass emission filter to a Quantix CCD camera (Photometrics, Tucson, AZ), which is equipped with a Kodak KAF 1400 charge-coupled device (grade 2, MPP) with 1,317 × 1,035 pixels and cooled to −25°C by a thermoelectric cooler. Image sequence acquisition (or pairs of images, in the case of ratiometric indicators) is controlled by a homemade program based on V for Windows software (Digital Optics, Auckland, New Zealand). To avoid bleaching of the probe and photodamage of the cells, the shuttered illumination was restricted to the periods when the images were taken. Signals were obtained by whole-image spatial integration of pixels over the confluent cells. The background image is automatically subtracted, pixel by pixel, from the loaded cell images. The value of the detected fluorescence was increased by applying 3 × 3 binning and a gain of 3.

**Determination of [Ca\(^{2+}\)]\(_i\).** [Ca\(^{2+}\)]\(_i\), was monitored using the fluorospect probe fura 2. The cells were loaded with fura 2 by incubation with the membrane-permeant acetoxyethyl (AM) ester form of the dye (2 \(\mu\)M from a 5 mM stock solution in DMSO) for 1 h at 37°C in the presence of 0.05% wt/vol Pluronic F-127. For excitation, 340/10- and 380/10-nm band-pass filters were used. Emission was recorded using a dichroic mirror type 72100 (>500-nm long-pass filter) and a 535/50-nm emission band-pass filter. Data collection time for an image was 5 s. Fura 2 was calibrated in vivo at the end of each experiment, according to the equation derived by Grynkiewicz et al. (26)

\[
[Ca^{2+}]_i = K_dR_{	ext{max}}(r - r_{	ext{min}})/(r_{	ext{max}} - r)
\]

where \(K_d\) is the dissociation constant of fura 2 for Ca\(^{2+}\) (135 mM) (75); \(R_{	ext{max}}\) is the maximum fluorescence intensity due to excitation at 380 nm (in the absence of Ca\(^{2+}\)) divided by the minimum fluorescence intensity at 380 nm (in the presence of saturating Ca\(^{2+}\)); \(r\) is the F\(_{340}/F\(_{380}\) fluorescence ratio; \(r_{	ext{max}}\) and \(r_{	ext{min}}\) are the F\(_{340}/F\(_{380}\) fluorescence ratios in the presence of saturating Ca\(^{2+}\) and in the absence of Ca\(^{2+}\), respectively; \(r_{\text{max}}\) was obtained by permeabilizing the cells with the Ca\(^{2+}\) ionophore ionomycin (10 \(\mu\)M from a 1 mM stock solution in ethanol) in the presence of 1.5 mM extracellular Ca\(^{2+}\). To obtain the minimum ratio \(r_{\text{min}}\) subsequently, the cells were exposed to a Ca\(^{2+}\)-free solution (containing 10 mM EGTA) with 10 \(\mu\)M ionomycin and BAPTA-AM (10 \(\mu\)M from a stock solution of 10 mM in DMSO) to buffer intracellular Ca\(^{2+}\).

**Determination of \(\Delta W_{\text{norm}}\).** \(\Delta W_{\text{norm}}\) was evaluated using the potentiometric indicator 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (12, 23, 60, 67). MDCK cells were loaded with JC-1 (10 \(\mu\)M from a 10 mM stock solution in DMSO) for 30 min at 37°C. JC-1 in its monomeric form emits fluorescence at 530 nm (green fluorescence) when excited at 490 nm, whereas the aggregates emit fluorescence at 590 nm (red fluorescence). Dual-emission ratiometric measurements were performed by manually changing the emission cube. The excitation was done through a 10-nm band-pass filter centred at 495 nm. JC-1 monomer fluorescence was collected through a >500-nm long-pass dichroic mirror and a 535/50-nm band-pass emission filter. The emission cube used to detect the J aggregates consisted of a >560-nm long-pass dichroic mirror and a 590/55-nm emission filter. Data collection time for an image was 5 s, and a neutral-density filter of 1.0 OD (Newport, Irvine, CA) was inserted in the excitation pathway. The mitochondrial uncoupler FCCP (10 \(\mu\)M) was added at the end of each experiment to determine the JC-1 emission ratio associated with a collapsed \(\Delta W_{\text{norm}}\). 2-DG (10 mM) was administered simultaneously with FCCP to rule out glycolytic ATP provision for the reverse action of the mitochondrial F\(_{1}\)F\(_{0}\)-ATPase. Results are presented in terms of a normalized ratio (R\(_{\text{norm}}\))

\[
R_{\text{norm}} = 100 [(R - R_{\text{FCCP}})/(R_{\text{control}} - R_{\text{FCCP}})]
\]

(2)

to compare experiments with different control values of the JC-1 emission ratio, R (F\(_{340}/F_{533}\)). R\(_{\text{control}}\) and R\(_{\text{FCCP}}\) are the emission ratios in control conditions and after the addition of FCCP plus 2-DG, respectively. Exposure of the cells to gramicidin D (10 \(\mu\)M) to depolarize the plasma membrane induced no changes in the JC-1 ratio in MDCK cells (n = 2; data not shown). This indicates that JC-1 fluorescence is insensitive to changes in cellular potential as stated in the literature (64).

**Determination of intracellular Na\(^+\) concentration.** Intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) was monitored using the fluorescent
probe sodium-binding benzo furan isothionate (SBFI). The cells were loaded with SBFI for 2 h at 37°C (2 μM from a 5 mM stock solution in DMSO, in the presence of 0.05% wt/vol Pluronic F-127). SBFI was used in dual-excitation ratiometric mode. For excitation, 340/10- and 380/10-mm band-pass filters were used. Emission was recorded using a dichroic mirror type 72100 (>500-nm long-pass filter) and a 535/50-mm band-pass emission filter. Data collection time for an image was 5 s. The calibration of the fluorescence signals of SBFI was accomplished by exposing the cells to various extracellular [Na+] in the presence of the ionophore gramicidin D (10 μM from a 2 mM stock solution in ethanol). A calibration curve was derived according to the procedure described by Zahler et al. (79). The ratio of the fluorescence signal of SBFI due to excitation at 340 nm (F340) over that at 380 nm (F380) was normalized as follows

\[ \bar{r} = \frac{(r - r_0)/(r_{90} - r_0)}{K_0/[Na] / (K_1 + [Na])} \]

where \( r_0 \) and \( r_{90} \) are the fluorescence ratios F340/F380 at 0 and 90 mM Na+, respectively. Experimental values were fitted via the Marquardt-Levenberg nonlinear least squares fit (51) according to

\[ \bar{r} = \frac{(r - r_0)}{(r_{90} - r_0)} \]

where \( K_0 \) and \( K_1 \) are fitting parameters.

**Laser-Scanning Confocal Microscopy**

The coverslips with MDCK cells were mounted into a holder and placed on the stage of the Zeiss LSM 510 META laser-scanning confocal microscopic (LSCM) system attached to an Axiovert 200 (motorized) frame (Zeiss). The microscope stage was equipped with a PeCon (model P, Erbach-Bach, Germany) heated specimen holder with an S-type incubator, all set at 37°C. To minimize temperature differences between sample and objective, a ×63 oil model objective heater (PeCon) was used. Cells were loaded with two fluorescent probes: Mito Tracker Green (MTG), a fluorescent probe that selectively stains mitochondria, and rhod 2, a Ca2+-sensitive probe. Loading of MDCK cells with MTG and rhod 2 occurred in two subsequent steps. At first, MTG (200 nM) was loaded into the cells for 30 min at 37°C. This was followed by incubation of the cells for 30 min at 37°C in a loading solution containing rhod 2 AM (4 μM), MTG (200 nM), and pluronic acid (0.025% wt/vol). Rhod 2-AM is a cell-permeant indicator that carries a delocalized positive charge. Therefore, it is taken up preferentially into polarized mitochondria. On hydrolysis of the ester moieties, the rhod 2-free acid remains trapped inside the mitochondria, where it reports increased [Ca2+]i by an increase in its fluorescence intensity. After being loaded, the cells were gently washed three times with normal saline solution.

Fluorescence measurements were performed with a ×63/1.4 Plan-Apochromat oil-immersion objective (Zeiss). MTG was excited by the Ar laser (488-nm line) and rhod 2 by the Green HeNe laser (543-nm line). Laser intensity was set at only 0.5% of the maximum level to minimize dye bleaching and to protect the cells against possible photodamage (44). Fluorescence emissions of MTG and rhod 2 were collected, respectively, via NFT490 and NFT545 dichroic mirrors and 525/25-nm and 590/25-nm barrier filters that came with the confocal microscope. The scanning speed was set to a pixel dwell time of 25.6 μs. Each 512 × 512-pixel image was averaged twice via software-selected repeated line scan mode to ameliorate the signal-to-noise ratio. The effective frame collection time was ∼33 s. All images were collected with a digital zoom factor of 2. The thickness of the optical slice was <1.4 μm in all experiments.

To monitor only the changes in [Ca2+]i during MI, rhod 2 fluorescence had to be corrected for nonmitochondrial rhod 2 signals, including the weak cytosolic rhod 2 staining and the pronounced staining of structures within the nuclei, presumably nuclear membranes (see Figs. 6C). Within the histogram of the MTG image, a threshold was chosen to retain only the high-end tail, excluding any saturated pixels. On visual inspection, resulting MTG intensities represented "well-delin-

**RESULTS**

**Influence of MI on [Ca2+]i in MDCK Cells**

To investigate whether MI (for details, see MATERIALS AND METHODS) had an effect on cytosolic Ca2+ levels, fura 2 fluorescence was monitored during a 60-min incubation period with metabolic inhibitors. In the first 20 min of MI, a profound increase in [Ca2+]i from 48 ± 2 to 631 ± 78 nM (n = 12) was seen (Fig. 1). This increase in cytosolic Ca2+ was due to an influx of extracellular Ca2+, because exposure to metabolic inhibitors in Ca2+-free medium did not evoke a significant increase in [Ca2+]i (n = 3; data not shown). Subsequently, a second-phase drop in [Ca2+]i occurred to a level of 118 ± 9 nM (n = 12) after 60 min of MI (Fig. 1). To unravel the mechanism underlying this second-phase decrease in cytosolic Ca2+, different Ca2+ efflux routes were checked: Ca2+ extrusion out of the cell and Ca2+ uptake into the intracellular organelles such as the ER and mitochondria.
Second-Phase Decrease in Cytosolic Ca\(^{2+}\) Is Not Due to Ca\(^{2+}\)-Extrusion Out of the Cell

MDCK cells possess two different types of Ca\(^{2+}\) extruders in their plasma membrane: Ca\(^{2+}\)-ATPases and NCEs (7, 47). Luciferine-luciferase-based ATP experiments revealed that intracellular ATP levels rapidly drop to only 9 ± 2% of control levels in 5 min of exposure to metabolic inhibitors. After a 20-min incubation period, only 1.8 ± 0.2% of the initial ATP content is left (n = 6, Fig. 2A). Furthermore, experiments (Fig. 2B) were performed with La\(^{3+}\), a known inhibitor of plasma membrane Ca\(^{2+}\)-pumps in different cell types (8, 9, 22, 33) including MDCK cells (43). Addition of La\(^{3+}\) did not change the typical cytosolic Ca\(^{2+}\) clearance after 20 min of MI. Therefore, it is unlikely that the ATP-dependent Ca\(^{2+}\) pumps account for the decrease in [Ca\(^{2+}\)]\(_i\) seen after 20 min of MI. To investigate whether the NCEs play a role in Ca\(^{2+}\) extrusion after 20 min of MI, SBFI experiments were performed to monitor [Na\(^{+}\)]\(_i\) in MDCK cells during MI (Fig. 3). [Na\(^{+}\)]\(_i\) rapidly increased during MI from 23 ± 3 mM in control conditions to 91 ± 4 mM (n = 7) after 60 min of MI. Because [Na\(^{+}\)]\(_i\) already increased by a factor greater than three after 20 min of MI, the ability of NCEs to extrude Ca\(^{2+}\) is strongly attenuated at that moment.

Mitochondria Take Up Cytosolic Ca\(^{2+}\) During MI

Buffering of high cytosolic Ca\(^{2+}\) levels can occur via Ca\(^{2+}\)-uptake into the ER via the thapsigargin-sensitive Ca\(^{2+}\)-ATPases and/or into the mitochondria via uniporter systems (4, 18, 27, 28, 30, 46). Because the presence of thapsigargin (1 µM), a well-known inhibitor of the ATPase responsible for Ca\(^{2+}\) uptake into the ER or SR, did not alter the typical biphasic behavior of [Ca\(^{2+}\)]\(_i\), during MI (Fig. 4), the possibility of mitochondrial uptake of cytosolic Ca\(^{2+}\) was further explored. Mitochondria take up Ca\(^{2+}\) via uniporter systems. These Ca\(^{2+}\) uniporters are driven by the Ca\(^{2+}\) electrochemical gradient, whose dominant component in mitochondria is the highly negative ΔΨ\(_m\) (18, 27, 29). ΔΨ\(_m\) is generated mainly by
H\(^+\) extrusion in the electron transport chain (ETC). However, when CN, a specific inhibitor of the cytochrome c oxidase complex, is used, the ETC is blocked and \(\Delta \Psi \text{m}\) is expected to collapse. \(\Delta \Psi \text{m}\) was evaluated with the fluorescent indicator JC-1. As a parameter for \(\Delta \Psi \text{m}\) the emission ratio \(F_{590}/F_{535}\) was used. To allow comparison of experiments with different control ratios, JC-1 ratios were normalized (see MATERIALS AND METHODS). Figure 5 illustrates the time course of the drop in \(\Delta \Psi \text{m}\) in response to CN and 2-DG incubation. \(\Delta \Psi \text{m}\) depolarized steadily to 12\(\pm\)4\% of control in 12 min. Thereafter, \(\Delta \Psi \text{m}\) declined further, but more slowly, to \(<\)1\% of control after 30 min of MI (n = 8). Because of this rapid loss of \(\Delta \Psi \text{m}\), it is rather unlikely that the mitochondrial uniporter is responsible for the second-phase cytosolic Ca\(^{2+}\) clearance (4, 39). To rule out any possible contribution of the mitochondrial uniporter, the protonophore FCCP (10 \(\mu\)M) was added in addition to the metabolic inhibitors to fully depolarize \(\Delta \Psi \text{m}\) (n = 3; data not shown). The second-phase decrease in [Ca\(^{2+}\)] persisted.

Mitochondrial Ca\(^{2+}\) Uptake During MI in MDCK Cells Occurs via the Mitochondrial Na\(^+\)/Ca\(^{2+}\) Exchanger Acting in Reverse Mode

In metabolically inhibited rat cardiomyocytes, it was proposed that the mitochondrial NCE might reverse (24). We hypothesized that the observed second-phase clearance of cytosolic Ca\(^{2+}\) in MDCK cells might be ascribed to the mitochondrial NCE acting in reverse mode. To test this hypothesis, MDCK cells were preincubated during 15 min in a Na\(^+\)-free solution (see MATERIALS AND METHODS) to deplete both mitochondria and the cytosol and thus to block the mitochondrial NCE. Subsequently, cells were exposed to MI under Na\(^+\)-free conditions. As depicted in Fig. 6A, [Na\(^+\)], dropped to 6 \(\pm\) 2 mM in the 15-min preincubation period in Na\(^+\)-free Ringer. After a subsequent 20-min period of Na\(^+\)-free MI, [Na\(^+\)], values were as low as 1 \(\pm\) 1 mM. This observation suggests that, after 20 min of MI, intracellular Na\(^+\) and presumably mitochondrial Na\(^+\) has decreased to levels where mitochondrial Na\(^+\)/Ca\(^{2+}\) exchange would be unlikely. The typical

Fig. 4. Changes in [Ca\(^{2+}\)], during MI in the presence of thapsigargin. The MDCK monolayers (n = 3) were preincubated during 6 min with thapsigargin (1 \(\mu\)M) and subsequently subjected to MI in the presence of thapsigargin. Values are means \(\pm\) SE.

Fig. 5. Effect of MI on the mitochondrial potential (\(\Delta \Psi \text{m}\)) in MDCK cells. \(\Delta \Psi \text{m}\) was assessed by the normalized ratio (R\(_{\text{norm}}\); see Eq. 2 in MATERIALS AND METHODS), which decreases on depolarization of the mitochondrial inner membrane. Values are means \(\pm\) SE from 8 different monolayers.

Fig. 6. Changes in [Na\(^+\)], and [Ca\(^{2+}\)], during MI in the absence of external Na\(^+\). A: decrease in [Na\(^+\)], during preincubation in Na\(^+\)-free Ringer and subsequent treatment with metabolic inhibitors in Na\(^+\)-free conditions is shown. Values are means \(\pm\) SE of 6 different monolayers. B: effect of MI on [Ca\(^{2+}\)], in the absence of external Na\(^+\). MDCK monolayers (n = 7) were incubated in Na\(^+\)-free solution for 15 min before and during treatment with metabolic inhibitors. Control tissues (C; n = 4) were exposed to Na\(^+\)-free solution without metabolic inhibitors during 75 min. Values are means \(\pm\) SE.
second-phase decrease in \([\text{Ca}^{2+}]\), seen after 20 min of MI was absent under these conditions (Fig. 6B), indicating the presumable \(\text{Na}^+\) dependence of the transport mechanism responsible for the removal of cytosolic \(\text{Ca}^{2+}\). Long-term exposure to \(\text{Na}^+\)-free solutions as such did not evoke any significant changes in \([\text{Ca}^{2+}]\), as indicated by the control trace in Fig. 6B. To further explore the hypothesis of mitochondrial NCE reversal, changes in \([\text{Ca}^{2+}]\), during MI were evaluated in the presence of CGP-37157, a specific inhibitor of the mitochondrial NCE (1, 3, 11, 13, 14, 54, 65). When CGP-37157 was administered during a 30-min preincubation period and subsequent exposure during 60 min in the presence of metabolic inhibitors, the second-phase drop in \([\text{Ca}^{2+}]\), was completely abolished (\(n = 8\), Fig. 7).

**Imaging Mitochondrial \(\text{Ca}^{2+}\) During MI with Confocal Microscopy**

To evaluate changes in mitochondrial \(\text{Ca}^{2+}\) content during MI, experiments were performed in MDCK cells loaded with both the mitochondrion-specific dye MTG and the \(\text{Ca}^{2+}\)-sensitive probe rhod 2. Wirelike mitochondria were observed in MTG-loaded cells in control conditions (Fig. 8A). Incubation with metabolic inhibitors induced conformational changes, resulting in the vesicle shape of the majority of the mitochondria (Fig. 8B). The changes in mitochondrial shape are related to MI rather than resulting from a time effect, because incubation in normal saline solution still revealed wirelike mitochondria after a 60-min incubation period (images not shown). Comparison of Fig. 8, B and C, illustrates that rhod 2 is mainly localized in mitochondria when loaded in MDCK cells with a weak staining of the cytosol. Moreover, a pronounced staining of structures within the nuclei of the cells, presumably nucleoli, was seen, as reported earlier by several other groups (3, 36, 57, 63). To resolve mitochondrial rhod 2 fluorescence (\(F_{\text{rhod 2, mito}}\)) from nonmitochondrial rhod 2 contributions, a “mask” procedure was used based on mitochondrial localization in the MTG image (for details, see MATERIALS AND METHODS). The resulting “mitochondrial” rhod 2 image (Fig. 8D) confirms colocalization of MTG and rhod 2 in mitochondria, because only rhod 2 intensities are retained that correspond to MTG-positive pixels in the MTG mask. Figure 9 clearly depicts that mitochondria indeed buffer cytosolic \(\text{Ca}^{2+}\) during MI in MDCK cells. During 60 min of MI, \(F_{\text{rhod 2, mito}}\) increased steadily to 346 ± 23% of the control level determined just before exposure to metabolic inhibitors (\(n = 5\)). The observed increase in \(F_{\text{rhod 2, mito}}\) during MI is underestimated, because open bars in Fig. 9 indicate that some mitochondrial rhod 2 fluorescence is lost throughout the 60-min protocol performed (\(n = 3\)). This loss of signal might be ascribed to either photobleaching of the probe or leakage of the rhod 2 dye out of the mitochondrial matrix. When MI was applied under \(\text{Na}^+\)-free conditions (after a preincubation period of 15 min in \(\text{Na}^+\)-free solution), only a minimal increase in \(F_{\text{rhod 2, mito}}\) was observed (\(n = 6\), pointing to the \(\text{Na}^+\) dependence of mitochondrial \(\text{Ca}^{2+}\) uptake. Furthermore, when MDCK cells were exposed to metabolic inhibitors in the presence of the specific inhibitor CGP-37157 (25 \(\mu\)M) of the mitochondrial NCE, the mitochondrial accumulation of cytosolic \(\text{Ca}^{2+}\) was substantially reduced (\(n = 5\)). The fact that \(F_{\text{rhod 2, mito}}\) is steadily increasing during MI and can be modulated by a specific mitochondrial agent, CGP-P37157, tends to confirm the mitochondrial origin of the rhod 2 fluorescence intensities obtained via the mask procedure applied.

**DISCUSSION**

In the present study, MI with CN and 2-DG was used as an experimental model to simulate ischemic cell injury. We examined the ability of MDCK cells to maintain cellular and mitochondrial \(\text{Ca}^{2+}\) homeostasis during MI.

**MI Induces a Transient Increase in Cytosolic \(\text{Ca}^{2+}\) in MDCK Cells**

Our data show biphasic behavior of \([\text{Ca}^{2+}]\), during MI in MDCK cells. The peak value of 631 ± 78 nM was reached after ~20 min of MI. Similarly, a previous study in confluent monolayers of MDCK cells reported that application of 5 mM CN and 5 mM 2-DG resulted in an increase in \([\text{Ca}^{2+}]\), from 112 ± 11 to 649 ± 99 nM in 15 min of MI, which was the maximum time interval investigated (52). We concluded that the increase in cytosolic \(\text{Ca}^{2+}\) was due to an influx of extracellular \(\text{Ca}^{2+}\) because \(\text{Ca}^{2+}\)-free MI did not evoke a substantial increase in \([\text{Ca}^{2+}]\), (data not shown). Moreover, thapsigargin experiments revealed that release of \(\text{Ca}^{2+}\) from the ER did not underlie the observed increase in \([\text{Ca}^{2+}]\), in the first phase of MI. When the ER was depleted for \(\text{Ca}^{2+}\) before MI during a preincubation period of 6 min with thapsigargin (1 \(\mu\)M), a well-known inhibitor of the ATPase responsible for \(\text{Ca}^{2+}\) uptake into ER, the typical biphasic behavior of \([\text{Ca}^{2+}]\), during subsequent MI was not altered (Fig. 4). Furthermore, the possibility of release of \(\text{Ca}^{2+}\) from mitochondrial stores in the first phase of MI seems unlikely, because the rhod 2 measurements in this study clearly demonstrate that mitochondrial \(\text{Ca}^{2+}\) levels increase during the first 20 min of MI (Fig. 9). Because the MI-induced rise in \([\text{Ca}^{2+}]\), was comparable in the absence (Fig. 6B) or presence (Fig. 1) of extracellular \(\text{Na}^+\), \(\text{Ca}^{2+}\) influx probably did not occur via the plasma membrane NCE. \(\text{Ca}^{2+}\) influx might be mediated via epithelial \(\text{Ca}^{2+}\) channels, e.g., via TRPV5 channels known to be present in the

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**Fig. 7.** Influence of CGP-37157 on \([\text{Ca}^{2+}]\), changes during MI in MDCK cells. CGP-37157 (25 \(\mu\)M) was added during a 30-min preincubation period and simultaneously with metabolic inhibitors during a subsequent 60-min incubation period. Values are means ± SE; \(n = 8\).
Ca\(^{2+}\)-transporting distal part of renal tubules (34). However, whether these Ca\(^{2+}\) channels are present in MDCK cells is unknown.

To our knowledge, the only previous study that reported a decline in \([\text{Ca}^{2+}]_i\) after an initial increase in renal cells exposed to ischemic conditions was performed in primary cultures of rat proximal tubular cells (10). In that study, 25% of cells exposed to glucose-free, anoxia \([\text{Ca}^{2+}]_i\) showed values that peaked to \(>1\ \mu\text{M}\) and then dropped rapidly to near \(500\ \text{nM}\) in \(\sim10\ \text{min}\). The observed partial recovery was associated with an extended period of survival.

In the present study, \([\text{Ca}^{2+}]_i\) was monitored by fura 2 ratio imaging. The \(K_d\) of fura 2, known to increase with intracellular acidification (40), and a given fluorescence ratio \(R\) will then correspond to a higher \([\text{Ca}^{2+}]_i\) value (Eq. 1). BCECF fluorescence experiments revealed that the intracellular pH value (pH\(_i\)) dropped in metabolically inhibited MDCK cells from \(7.35 \pm 0.03\) to \(7 \pm 0.03\) in \(\sim6\ \text{min}\) (data not shown). Because a constant \(K_d\) was used in our calculations, the reported \([\text{Ca}^{2+}]_i\) values might underestimate real \([\text{Ca}^{2+}]_i\) values.

**Second-Phase Ca\(^{2+}\) Clearance During MI Is Not Due to Ca\(^{2+}\) Extrusion Out of the Cell**

The direction of Ca\(^{2+}\) movement by the plasma membrane NCE is determined by several variables, including membrane potential as well as intracellular and extracellular concentrations of both Na\(^+\) and Ca\(^{2+}\). Both membrane depolarization and an increase in \([\text{Na}^+]_i\) are expected to occur during ischemia, and both of these factors favour exchanger-mediated cellular Ca\(^{2+}\) entry rather than exit. We found that \([\text{Na}^+]_i\) steadily increased more than three times times during 20 min of MI, which does not favor Ca\(^{2+}\) extrusion out of the cells after that time. Moreover, the NCE may reverse in ATP-depleted cells, as already reported in several cell types, including cardiac myocytes (31), neurons (66), and human keratinocytes (42).

In this study, incubation with metabolic inhibitors rapidly induced depletion of cellular ATP levels. After only 5 min of MI, cellular ATP levels dropped to <10% of control ATP levels. This result is consistent with previous reports on MDCK cells (16, 21, 72). Earlier reports indicate that the affinity for ATP of isolated plasma membrane Ca\(^{2+}\)-ATPases...
Fig. 9. Changes in mitochondrial rhod 2 fluorescence intensities (F

\[ \text{rhod}_2 \text{,mito} \]) during MI in MDCK cells. F

\[ \text{rhod}_2 \text{,mito} \] is expressed as a percentage of control levels as determined just before exposure to metabolic inhibitors (time 0). Open bars, decrease in F

\[ \text{rhod}_2 \text{,mito} \] as a function of time when the cells are kept in normal saline solution without metabolic inhibitors (n = 3); filled bars, steady increase in F

\[ \text{rhod}_2 \text{,mito} \] in cells (n = 5) after 20, 40, and 60 min of exposure to metabolic inhibitors; gray bars, F

\[ \text{rhod}_2 \text{,mito} \] in monolayers (n = 6) incubated in Na

\[ \text{H}^+ \text{-free solution} \] 15 min before and during treatment with metabolic inhibitors; striped bars, results for monolayers (n = 5) treated with CGP-37157 (25 nM) during a 30-min preincubation period and simultaneously with metabolic inhibitors during a subsequent 60-min incubation period. (PMCA) is very high (55, 56): a high-affinity site, normally assumed to be the catalytic site, has a Michaelis constant (K

\[ \text{M} \]) as low as 1–2 μM, and the low-affinity site has a K

\[ \text{M} \] between 150 and 400 μM (9). However, when K

\[ \text{M} \] values for ATP were determined in membrane vesicles derived from rabbit proximal tubules (76) and rat kidney cortex (73), much higher values were obtained: 0.6 and 0.2 mM, respectively. Assuming that the cellular ATP concentration ([ATP]i) in MDCK cells in control conditions was near 2.4 mM as determined by Lynch and Balaban (50) for MDCK cells, a drop to 2% of the initial ATP content after 20 min of MI (Fig. 2A) yields a residual [ATP]i value near 0.048 mM. This value is still considerably lower than the above-mentioned K

\[ \text{M} \] values for ATP of renal PMCs. Moreover, because ADP has been found to inhibit PMCA activity in membrane vesicles derived from rabbit proximal tubules (76) and, cellular ADP as well as AMP levels are known to increase in ischemia (49, 77), the activity of PMCs in metabolically inhibited MDCK cells might be additionally reduced. Furthermore, addition of La

\[ 3+ \], a known inhibitor of plasma membrane Ca

\[ 2+ \] pumps (8, 9, 33), did not change the typical cytosolic Ca

\[ 2+ \] clearance after 20 min of MI (Fig. 2B). Therefore, Ca

\[ 2+ \] extrusion via the PMCs is unlikely after 20 min of MI.

Ca

\[ 2+ \] Uptake in Depolarized Mitochondria via Na

\[ /Ca

\[ 2+ \] Exchanger Acting in Reverse Mode

Mitochondria can accumulate enormous quantities of Ca

\[ 2+ \] (3, 17, 61). In renal mammalian cells, the mean mitochondrial volume is 25% of the combined cytosolic and nuclear volumes (20). Mitochondria are key players in clearing large loads of cytosolic Ca

\[ 2+ \] by their fast, high capacity and reversible Ca

\[ 2+ \] sequestration properties (3, 18, 58, 70).

The normal route for mitochondrial Ca

\[ 2+ \] uptake in mitochondria is the ΔΨ

\[ m \]-dependent Ca

\[ 2+ \] uniporter, as highlighted by the fact that experimental collapse of ΔΨ

\[ m \] by mitochondrial inhibitors or uncouplers prevents mitochondrial Ca

\[ 2+ \] accumulation (32, 35, 41, 59, 62). In the present study, mitochondrial Ca

\[ 2+ \] uptake via the ΔΨ

\[ m \]-dependent uniporter is limited to the first 25–30 min of MI, because R

\[ \text{norm} \] decreased to <10% of control in 15 min and dropped to near-zero values after 30 min of MI (Fig. 5). However, our data show that in metabolically inhibited renal epithelial MDCK cells, the mitochondrial Ca

\[ 2+ \] content steadily increased during the 60-min incubation period with metabolic inhibitors (Fig. 9). Because the second-phase decrease in [Ca

\[ 2+ \] ]i was abolished and the increase in mitochondrial Ca

\[ 2+ \] content was nearly prevented when cells were depleted of Na

\[ + \] before and during induction of MI, it was concluded that mitochondria take up cytosolic Ca

\[ 2+ \] via an Na

\[ - \]-dependent transport mechanism. Furthermore, the pronounced inhibition of the increase in [Ca

\[ 2+ \] ]i when MI was applied in the presence of CGP-37157 provided evidence that the route of mitochondrial Ca

\[ 2+ \] entry during MI is the NCE. The idea of mitochondrial Ca

\[ 2+ \] loading by reversal of the NCE was proposed earlier in studies in isolated beef heart mitochondria (38) and in intact cardiomyocytes exposed to 2.5 mM KCN in glucose-free medium (24, 25). Jung et al. (38) treated mitochondria with rotenone and oligomycin to partially abolish ΔΨ

\[ m \], applied the blocker ruthenium red to inhibit the Ca

\[ 2+ \] uniporter, and challenged subsequently with Ca

\[ 2+ \] (13 μM). Only a minimal mitochondrial Ca

\[ 2+ \] uptake was observed. To dissipate the remaining ΔΨ

\[ m \] completely, the uncoupler FCCP was applied subsequently, leading to a 4.6-fold increase in the Ca

\[ 2+ \] influx rate. This FCCP-stimulated entry of Ca

\[ 2+ \] was 98% inhibited by the omission of Na

\[ + \], which is consistent with the abolition of mitochondrial Ca

\[ 2+ \] uptake in our Na

\[ + \]-free MI experiments.

To understand the observed reversal of the mitochondrial NCE, the following speculative conceptual model can be suggested. In this model, electrical as well as chemical driving forces for both Ca

\[ 2+ \] and Na

\[ + \] are considered. Because some investigators proposed that the stoichiometry of the mitochondrial NCE may be closer to 3Na

\[ + \]/1Ca

\[ 2+ \] than 2Na

\[ + \]/1Ca

\[ 2+ \] (4, 27, 38), elimination of ΔΨ

\[ m \] during MI removes the electrical driving force for Ca

\[ 2+ \] efflux via the putative electrogenic NCE. The loss of ΔΨ

\[ m \] allows for a reverse action of the NCEs. In general, resting values of [Ca

\[ 2+ \] ]i are comparable to those in the cytoplasm (3, 18) or are lower, as demonstrated in cardiomyocytes (5, 53). The observed increase in [Ca

\[ 2+ \] ]i in our experiments, from ~50 nM to values of >600 nM, induced a chemical driving force for Ca

\[ 2+ \] entry into the mitochondrial matrix. Therefore, the mitochondrial NCE in reverse mode is thought to be driven by the Ca

\[ 2+ \] gradient in the initial phase of the [Ca

\[ 2+ \] ]i decline. Despite the large [Na

\[ + \]i increase, the mitochondrial Na

\[ + \] gradient ([Na

\[ + \]i]/[Na

\[ + \]o]) may be surpassed by the larger Ca

\[ 2+ \] gradient (in the first 20 min of MI, [Ca

\[ 2+ \] o]), increased >10-fold, whereas [Ca

\[ 2+ \] o] only doubled). Moreover, in isolated heart mitochondria, it was demonstrated that [Na

\[ + \]o] increases with [Na

\[ + \]i] and that the Na

\[ + \] gradient across the internal mitochondrial membrane is significantly lower for nonrespiring vs. respiring mitochondria (37). However, the more Ca

\[ 2+ \] is taken up by the mitochondria, the more the chemical driving force for Ca

\[ 2+ \] weakens. To allow the observed decrease in [Ca

\[ 2+ \] ]i, values near 120 nM after 60 min of MI (Fig. 1), whereas F

\[ \text{rhod}_2 \text{,mito} \] signals increased almost to a factor of four (Fig. 9), one should hypothesize that the Na

\[ + \] gradient has to control Ca

\[ 2+ \] uptake via the NCE. This is only
possible if the intramitochondrial Na\(^+\) content \([\text{Na}^+]_m\) was augmented sufficiently to provide abundant Na\(^+\) for exchange with entering cytosolic Ca\(^{2+}\). However, the exact mechanism for the reverse action of the mitochondrial NCE remains to be elucidated.

Bernardi (4) described the existence of Mg\(^{2+}\)-modulated inner membrane channels with selectivity for Na\(^+\). Whether these Na\(^+\) channels contribute to mitochondrial Na\(^+\) loading during MI seems unlikely, because these channels are inhibited by nanomolar concentrations of Mg\(^{2+}\) and by acidification, which is generally seen in ischemic conditions. Moreover, as expected, inward Na\(^+\) fluxes through these channels are known to decrease with mitochondrial depolarization (4).

Apart from the NCE, mitochondria in kidney tissue have a Na\(^+\)-independent Ca\(^{2+}\) exchanger as the primary Ca\(^{2+}\) efflux pathway (27, 61). It is unlikely to assume a contribution of this exchanger (in reverse mode) in the mitochondrial Ca\(^{2+}\) accumulation seen during MI, because of 1) its Na\(^+\) independency, in contrast to the Na\(^+\) dependency of mitochondrial Ca\(^{2+}\) uptake in our experiments; and 2) the inhibition of this transport mechanism by CN\(^-\) (30, 78).

**Is Mitochondrial Ca\(^{2+}\) Uptake Beneficial for Cells in Ischemic Conditions?**

Because mitochondria have the capacity to take up huge amounts of Ca\(^{2+}\), they can remove toxic levels of Ca\(^{2+}\) from the cytosol. However, in some circumstances, mitochondrial Ca\(^{2+}\) uptake can switch from a useful physiological regulatory mechanism to a potentially harmful process that can initiate the progression toward cell death (18). Indeed, accumulating evidence suggests that \([\text{Ca}^{2+}]_m\) may play a critical role in ischemia-reperfusion injury (59). For example, \([\text{Ca}^{2+}]_m\) rises significantly during heart ischemia (2), and the magnitude of this rise determines the outcome of ischemia-reoxygenation (53). Mitochondrial Ca\(^{2+}\) overload can initiate opening of the mitochondrial permeability transition pore (MPTP) and cause cell death by either energetic collapse, ATP depletion, and necrotic cell death or by initiating mitochondrial swelling, cytochrome c release, and apoptotic cascades (1, 46, 48, 48). The MPTP is a large-conductance pore that forms under pathological conditions in the inner mitochondrial membrane. The channel is opened by a combination of high \([\text{Ca}^{2+}]_m\), oxidative stress, ATP depletion, high inorganic phosphate, and mitochondrial depolarization (15, 69). MPTP might play a major role in reperfusion injury rather than during the ischemic period, because the conditions needed for opening of the MPTP are exactly those that occur during reperfusion (69). Moreover, ischemia-induced intracellular acidification might prevent opening of the MPTP because opening is greatly inhibited at intracellular pH values <7 (69). In the present study, opening of the MPTP during mitochondrial Ca\(^{2+}\) accumulation in metabolically inhibited MDCK cells probably did not occur within the time course of our experimental protocol of 60 min, because MI induced a substantial intracellular acidification in MDCK cells (pH, dropped from 7.35 ± 0.03 to 7 ± 0.03, \(n = 7\); data not shown). Furthermore, no substantial rhod 2 release was seen, although rhod 2 is known to be released through the MPTP in isolated mitochondria (5).

**Summary**

The above results demonstrate that 1) mitochondria take up Ca\(^{2+}\) during MI despite their fully collapsed \(\Delta \Psi_m\); and 2) Ca\(^{2+}\) entry into mitochondria occurs via the NCE, whereas the \(\Delta \Psi_m\)-dependent unipporter is inactive. Up to now, the proposed mechanism of Ca\(^{2+}\) uptake into deenergized mitochondria via the reverse action of the mitochondrial NCE was only described for cardiomyocytes (24, 38). Our observations demonstrate that important alterations occur in mitochondrial Ca\(^{2+}\) transport pathways of metabolically inhibited distal renal epithelial cells: Ca\(^{2+}\) entry occurs via the NCE (the normal Ca\(^{2+}\) efflux pathway), whereas the Ca\(^{2+}\) unipporter (the normal influx route) is inactive.

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**REFERENCES**


