Metabolic inhibition-induced transient Ca\textsuperscript{2+} increase depends on mitochondria in a human proximal renal cell line

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Caplanusi A, Fuller AJ, Gonzalez-Villalobos RA, Hammond TG, Navar LG. Metabolic inhibition-induced transient Ca\textsuperscript{2+} increase depends on mitochondria in a human proximal renal cell line. Am J Physiol Renal Physiol 293: F533–F540, 2007. First published May 23, 2007; doi:10.1152/ajprenal.00030.2007.—During ischemia or hypoxia an increase in intracellular cytosolic Ca\textsuperscript{2+} induces deleterious events but is also implicated in signaling processes triggered in such conditions. In MDCK cells (distal tubular origin), it was shown that mitochondria confer protection during metabolic inhibition (MI), by buffering the Ca\textsuperscript{2+} overload via mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX). To further assess this process in cells of human origin, human cortical renal epithelial cells (proximal tubular origin) were subjected to MI and changes in cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{i}), Na\textsuperscript{+}, and ATP concentrations were monitored. MI was accomplished with both antimycin A and 2-deoxyglucose and induced a 3.5-fold increase in [Ca\textsuperscript{2+}]\textsubscript{i}, reaching 136.5 ± 15.8 nM in the first 3.45 min. Subsequently [Ca\textsuperscript{2+}]\textsubscript{i} dropped and stabilized at 62.7 ± 7.3 nM by 30 min. The first phase of the transient increase was La\textsuperscript{3+} sensitive, not influenced by diltiazem, and abolished when mitochondria were denervegorized with the protonophore carbonylcyanide m-trifluoromethoxyphenylhydrazone. The subsequent recovery phase was impaired in a Na\textsuperscript{+}-free medium and weakened when the mitochondrial NCX was blocked with 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157). Thus Ca\textsuperscript{2+} entry is likely mediated by store-operated Ca\textsuperscript{2+} channels and depends on energized mitochondria, whereas [Ca\textsuperscript{2+}]\textsubscript{i} recovery relied partially on the activity of mitochondrial NCX. These results indicate a possible mitochondrial-mediated signaling process triggered by MI, support the hypothesis that mitochondrial NCX has an important role in the Ca\textsuperscript{2+} clearance, and overall suggest that mitochondria play a preponderant role in the regulation of responses to MI in human renal epithelial cells.

Ca\textsuperscript{2+} influx; energized mitochondria; mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger; thapsigargin; CGP-37157

INTRACELLULAR Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) homeostasis depends on the activity of many cellular components including the Ca-ATPase located in the plasma membrane and in the endoplasmic reticulum (ER) membranes. Following ischemia, the ATP level drops rapidly and the efficiency of ATP-fueled Ca\textsuperscript{2+} pumps to clear Ca\textsuperscript{2+} is reduced, leading to increases in [Ca\textsuperscript{2+}]\textsubscript{i}. Hence the Ca\textsuperscript{2+} overload elicited by hypoxia is partially due to the gradual reduction in ATP levels (4, 28, 39). However, there is evidence that the [Ca\textsuperscript{2+}]\textsubscript{i} increase during acute hypoxic injury or metabolic inhibition (MI) is implicated in complex signaling processes (55). In regard to the kidney, the renal proximal epithelium is one of the main targets of ischemia (27), and Ca\textsuperscript{2+} overload is considered to have a major role in mediating ischemic damage at the renal cellular level (44). Therefore, the understanding of the basic mechanisms of Ca\textsuperscript{2+} dynamics is essential in designing protective strategies against hypoxic-ischemic injury.

The ability to recover after an ischemic insult is associated with many processes, including prompt reductions in the ischemia-induced [Ca\textsuperscript{2+}]\textsubscript{i}, increase (35, 40). In renal epithelial cells, restoring the low [Ca\textsuperscript{2+}]\textsubscript{i} seems to be an important factor that increases the resistance against MI (2, 7, 46). Mitochondria are able to buffer substantial quantities of Ca\textsuperscript{2+} under physiological and pathophysiological conditions and have an important role in maintaining intracellular Ca\textsuperscript{2+} homeostasis (12, 37). Classically, the Ca\textsuperscript{2+} buffering capacity of mitochondria relies on the mitochondrial Ca\textsuperscript{2+} uniporter, whose activity is triggered by a rise in [Ca\textsuperscript{2+}]\textsubscript{i}, and depends on the high inner mitochondrial membrane potential (ΔΨ). However, in particular physiological conditions, when ΔΨ is reduced or collapsed (i.e., during MI), other structures could be involved in mitochondrial Ca\textsuperscript{2+} buffering. Recent reports show that mitochondria protect the renal epithelial cell against Ca\textsuperscript{2+} overload during MI by the aim of mitochondrial Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX) (2, 46). These studies were carried out in MDCK cells and demonstrated that MI induces a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i}, whose recovery phase is mediated by the mitochondrial NCX working in the entry mode. The driving force of Ca\textsuperscript{2+} entry into mitochondria via NCX is likely sustained by an MI-induced increase in intracellular Na\textsuperscript{+} concentration ([Na\textsuperscript{+}]). The reversal of mitochondrial NCX during hypoxia was reported also in rat cardiomyocytes (16, 17). However, the responses in proximal tubular cells, which are thought to have much higher metabolic activity and therefore are more vulnerable to ischemic injury, have not been evaluated previously.

The aims of the present work were to investigate whether metabolically inhibited human renal proximal tubular cells exhibit a similar transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} and to determine the involvement of mitochondria in the MI-induced processes. Human cortical renal epithelial cells (HCRE) (3, 9, 25), which possess the characteristic brush border markers of the proximal tubule and have been used in the past as a model for this segment, were selected. Inhibitors of ATP production were
utilized because they have been used extensively to study ischemic injury in polarized epithelial cells (2, 11, 28, 46). MI, which mimics the ischemic process, was induced with both antimycin A-a blocker of complex III of mitochondrial electron transport chain (45) and 2-deoxyglucose, which blocks the glycolytic pathway in a glucose-free medium (48). To test the role of mitochondria in the [Ca$^{2+}$]i time course during MI, two compounds were used: 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP-37157), a benzodiazepine compound widely used as a specific blocker of mitochondrial NCX (8, 10, 32), and the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which collapses the inner mitochondrial membrane potential.

**MATERIALS AND METHODS**

Cells. SV-40 immortalized HCRE cells (primary cells obtained from Clonetics prior to immortalization) were used in this study. HCRE cells were previously characterized and used as a model of renal proximal epithelial cells (3, 9, 25). They show proximal tubular features including proximal tubular enzyme markers (γ-glutamyl transpeptidase, alkaline phosphatase, and leucine aminopeptidase), form a polarized monolayer with apical microvilli, and express the proximal tubular receptors cubulin and megalin (25). They maintain active sodium-dependent transport of phosphate and glucose (3). The cells were cultured in conventional T75 flasks in an incubator with humidified atmosphere and 5% CO₂, by using DMEM/F12 (GIBCO/Invitrogen, Carlsbad, CA) supplemented with heat-inactivated fetal calf serum (10%) and an antibiotic cocktail: Ciprofloxacin (Bayer, Molecular Probes (Eugene, OR)) supplemented with 0.05% wt/vol Pluronic F-127. SBFI was used in a dual excitation ratiometric mode. The ratiometric signal was achieved by exposing the cells to various extracellular Na⁺ concentrations in the presence of the ionophore gramicidin D (10 μM) at the end of each experiment. A calibration curve was derived according to the procedure described by Zahler et al. (56).

**Cellular ATP content.** Fully confluent HCRE monolayers were used for the determination of the ATP content. The coverslips were gently washed several times with normal saline solution and afterward subjected to MI for various time intervals ranging from 5 to 30 min. ATP measurements were performed with a luciferin-luciferase-based assay kit (Sigma, St. Louis, MO). The reaction buffer contained 150 μg/ml luciferin, 1.25 μg/ml luciferase, 5 mM MgSO₄, 1 mM dithiothreitol, 25 mM tricine, 0.1 mM EDTA, and 0.1 mM azide, at pH 7.8. The cells were solubilized in 450 μl of Somatic cell ATP-releasing agent (Sigma) for 30 s, and 50 μl of cell extract was added to 450 μl of reaction buffer. ATP levels were measured with a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). The results are expressed as percent change compared with control. The control ATP values were given by monolayers (2 coverslips per point) processed in the same way but not subjected to MI.

**Experimental protocols.** MI was accomplished by inhibiting simultaneously the mitochondrial respiration and the glycolytic pathway by using both antimycin A and 2-deoxyglucose, respectively. The MI effects in terms of [Ca$^{2+}$]i, [Na$^{+}$]i, and ATP concentration were investigated during 30-min exposures. [Ca$^{2+}$]i and ATP concentration were further monitored for a 10-min washout period, when the metabolic inhibitors containing solution were replaced by a normal saline solution, which was followed by another 15 min of MI. The MI effects on [Ca$^{2+}$]i either in the presence of various compounds or in a Ca$^{2+}$-free medium as well as a Na$^{+}$-free medium were investigated during 30-min exposures.

**Solutions and chemicals.** HCRE cells were bathed in a normal saline solution containing (in mM) 140 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgSO₄, 10 HEPES, and 5.5 glucose, pH adjusted to 7.4 with Tris. MI was accomplished with a solution containing (in mM) 135 NaCl, 5 KCl, 1.5 CaCl₂, 1 MgSO₄, 10 HEPES, and 10 2-deoxyglucose (pH 7.4 with Tris); 10 μM antimycin A was added to the solution before use. Ca$^{2+}$-free solutions contained 10 mM EGTA. N-methyl-d-glucamine replaced Na⁺ in the Na$^{+}$-free solutions. Fura 2-AM, SBFI-AM, BAPEA, and phosphonic F-127 were obtained from Invitrogen-Molecular Probes (Eugene, OR). FCCP, iodide, thapsigargin, gramicidin D, EGTA, and LaCl₃ were provided by Sigma. CGP-37157 was obtained from Tocris (Bristol, UK).

**Statistics.** Data values from n different monolayers are given as means ± SE. Statistical comparisons were made with the Mann-Whitney U-test when applicable. A value of P < 0.01 was considered significant.

**RESULTS**

MI was achieved by using simultaneously both metabolic inhibitors: antimycin A and 2-deoxyglucose. This treatment induces in HCRE cells an important energetic deficit. As depicted in Fig. 1, as a consequence of MI, the ATP levels dropped to 35.6% from the control in 5 min, to 13.1% in 10 min, and to 1.5% of control after 30 min of MI. On washout, the ATP level recovered after 10 min to ~17% of the control and during the second exposure to MI, the ATP level dropped
to 0.9% from the control. However, we verified that the cells were alive at the end of the 30-min MI period by their capacity to recover and by the Trypan blue exclusion test (which showed >98% viability after 30 min of MI). Moreover, the cells did not detach spontaneously from the monolayer after 30 min of MI, which is also a reflection of viability (26).

In HCRE cells MI induced a transient increase in [Ca^{2+}]_i (○, Fig. 2). The initial increase in [Ca^{2+}]_i, occurred within 3.45 min (from 39.6 to 136.5 ± 15.8 nM) and was followed by a recovery phase, in which [Ca^{2+}]_i partially recovered and stabilized at 62.7 ± 7.3 nM. During the washout period [Ca^{2+}]_i recovered to 39.4 ± 8.1 nM in 10 min. It is worthwhile to note that despite the considerable energetic deficit the [Ca^{2+}]_i transient occurred in the presence of nonnegligible amounts of ATP. A second exposure to MI did not induce a transient increase in [Ca^{2+}]_i, (peak type) but a continuous increase up to a level of 74.9 ± 11.9 nM.

To reveal the factors responsible for the generation of the transient increase in [Ca^{2+}]_i, first we established the source of Ca^{2+} (extracellular vs. intracellular). When HCRE cells were subjected to MI in a Ca^{2+}-free medium, the MI-induced increase in [Ca^{2+}]_i was reduced, accounting only for ~17 nM, from 4.4 ± 1.4 to 21.2 ± 2.8 nM (○, Fig. 2) and suggesting that the increase in [Ca^{2+}]_i, was mainly originated from outside the cells. Secondly, to rule out the possibility that this transient elevation in [Ca^{2+}]_i, could have been induced only by antimycin A or 2-deoxyglucose alone, [Ca^{2+}]_i, was monitored in the presence of these inhibitors separately. As shown in Fig. 3, neither in the case of antimycin A (△) nor in the case of 2-deoxyglucose alone (▲) was a transient increase in [Ca^{2+}]_i, noticed.

Which Ca^{2+} entry pathways could be involved in the noticed transient [Ca^{2+}]_i, elevation? Taking into account that the major route for Ca^{2+} entry in nonexcitable cells is represented by the store-operated Ca^{2+} channels (SOCs) (37) and a previous report suggesting that these channels mediate hypoxia induced Ca^{2+} entry in proximal tubule cells (41), MI was applied in the presence of La^{3+}, a blocker of SOCs. Figure 4A shows that MI induced a significantly (P < 0.01) smaller initial transient increase in [Ca^{2+}]_i, in the presence of La^{3+}, of only ~30 nM compared with the control of ~100 nM (○) (Fig. 2). Importantly, the transient increase in [Ca^{2+}]_i, was still distinct. These results suggest that SOCs are among the Ca^{2+} entry pathways in HCRE cells subjected to MI. Furthermore, we also observed that diltiazem, a blocker of L-type voltage-operated Ca^{2+} channels (VOCs), did not modify the transient [Ca^{2+}]_i, rise (n = 3; data not shown).

Interestingly, the transient increase in [Ca^{2+}]_i, was blocked by FCCP, a protonophore that dissipates the proton gradient across the inner mitochondrial membrane. When cells were preincubated with FCCP for 5 min and then subjected to MI in the continuous presence of FCCP, the transient increase did not occur and [Ca^{2+}]_i, steadily increased during MI, reaching 98.6 ± 18.1 nM at the end of the MI period (Fig. 4B). Notice that when FCCP is applied, during the preincubation period, a peak-type response in [Ca^{2+}]_i, occurs, which is likely correlated with the inactivation of the mitochondrial Ca^{2+} uniporter.
applied, MI induced a slight increase in \([\text{Ca}^{2+}]_i\), but exposure to MI, the cells were preincubated with FCCP for 10 min, followed by a decrease in \([\text{Ca}^{2+}]_i\) to 19.7 nM, which was reached in 2.5 min. This increase was followed by an incomplete recovery in \([\text{Ca}^{2+}]_i\), reaching 107.3 ± 6.3 nM at the end of the observation period. To facilitate the comparison, in Fig. 7 is also shown the control experiment: the time course of \([\text{Ca}^{2+}]_i\) in the absence of CGP-37157 (○).

To investigate the ability of mitochondrial NCX to reverse 

Furthermore, we focused on the recovery phase of the 

Our data show a biphasic behavior of \([\text{Ca}^{2+}]_i\), during MI in HCRE cells (Fig. 2). This feature of the \([\text{Ca}^{2+}]_i\) response has also been observed in MDCK cells (46) and in primary cultures of rat proximal tubular epithelial cells (7). In both cases, the \([\text{Ca}^{2+}]_i\) recovery during MI was shown to have a protective effect against ischemic damage. Neither the mitochondrial respiration arrest with antimycin A nor the glycolysis blockade with 2-deoxyglucose induced a transient increase in \([\text{Ca}^{2+}]_i\), (Fig. 3), ruling out a possible effect of either agent acting individually and suggest that only their combined effects in-

**Fig. 5.** Changes in cytosolic Na\(^+\) concentration ([Na\(^+\)]\(_{\text{i}}\)) during MI in a normal Na\(^+\) environment (○), \(n = 5\); in the absence of external Na\(^+\) (●), \(n = 4\); and in the absence of Na\(^+\) and the presence of CGP-37157 (△), \(n = 6\). For the experiments performed in the absence of Na\(^+\), the cells were incubated in a Na\(^+\)-free medium (without or with CGP-37157) for 10 min before and during the treatment with metabolic inhibitors (not shown). [Na\(^+\)]\(_{\text{i}}\) values are indicated as means ± SE. SE values are depicted in dotted lines.

**Fig. 4.** \([\text{Ca}^{2+}]_i\), changes during MI in HCRE cells. A: in the presence of 0.2 mM LaCl\(_3\). The peak value of \([\text{Ca}^{2+}]_i\) is significantly decreased compared with the control, \(P < 0.01\), Mann–Whitney U-test, \(n = 4\). B: in the presence of 1 μM carbonyl cyanide m-trifluoromethoxyphenylhydrazone (FCCP). Before the exposure to MI, the cells were preincubated with FCCP for 10 min, \(n = 4\).
Inhibit the \( \text{Ca}^{2+} \) increase induced by hypoxia. In renal epithelial distal cells neither verapamil nor nifedipine, VOCs blockers, could inhibit the \( \text{Ca}^{2+} \) increase induced by simulated ischemia (34). The present report suggests that the MI-induced \( \text{Ca}^{2+} \) entry in HCRE cells is mediated by SOCs. La\(^{3+}\), a SOC blocker (29), significantly decreased the \( \text{Ca}^{2+} \) entry (Fig. 4A). Moreover, no modification in the \( \text{[Ca}^{2+}]_i \) time course during MI occurred in HCRE cells in the presence of diltiazem, a blocker of VOCs; thus it is unlikely that these channels mediate the \( \text{Ca}^{2+} \) entry. Although the contribution of other calcium channels, such as the La\(^{3+}\)-sensitive transient receptor potential channels, to \( \text{Ca}^{2+} \) entry in HCRE cells during MI cannot be excluded, SOCs remain as the main candidates. Indirectly, this conclusion is further supported by the fact that after the depletion of ER \( \text{Ca}^{2+} \), MI failed to induce an increase in \( \text{[Ca}^{2+}]_i \) (Fig. 6A). Classically, \( \text{Ca}^{2+} \) entry via SOCs is triggered by the ER \( \text{Ca}^{2+} \) release (38). After the thapsigargin preincubation period, the ER \( \text{Ca}^{2+} \) stores are already depleted, and the \( \text{[Ca}^{2+}]_i \) level is the consequence of a new equilibrium between \( \text{Ca}^{2+} \) entry and \( \text{Ca}^{2+} \) extrusion out of the cells. Moreover, it was reported that the activity of ER \( \text{Ca}^{2+} \) pumps controls the gating of SOCs (29). Another argument in favor of SOCs as mediators of \( \text{Ca}^{2+} \) entry in HCRE cells subjected to MI relies on the fact that mitochondria can take up \( \text{Ca}^{2+} \) entering this way (20, 22).

During the second exposure to MI, the transient increase in \( \text{[Ca}^{2+}]_i \) did not occur (Fig. 2). Most likely this happens as a consequence of the impairment of mitochondrial signaling capacity and/or the ATP depletion status (~17% from the control) (Fig. 1). The connection between SOCs-mediated \( \text{Ca}^{2+} \) entry and ATP depletion was established in lymphocytes (33), in HeLa, Jurkat, and Erlich ascites tumor cells as well in hepatocytes (14). In these two studies, ATP depletion was proven to inhibit the capacitative \( \text{Ca}^{2+} \) entry. Moreover, in line with these findings, ADP was shown to inhibit \( \text{Ca}^{2+} \) release-activated \( \text{Ca}^{2+} \) current in a basophilic cell line (24).

**Fig. 6. [Ca\(^{2+}\)]\(_i\) changes during MI in HCRE cells. A: in the presence of thapsigargin, the cells were preincubated for 15 min with 1 \( \mu \text{M} \) thapsigargin before exposure to MI (not shown), \( n = 7 \). B: in the absence of external Na\(^+\), the cells were incubated in a Na\(^+\)-free medium for 10 min before and during treatment with metabolic inhibitors, \( n = 4 \). [Ca\(^{2+}\)]\(_i\) values are indicated as means ± SE. SE values are depicted in dotted lines.**

MI-induced \([\text{Ca}^{2+}]_i\) increase is mainly due to the \( \text{Ca}^{2+} \) influx. In the present experiments the \( \text{Ca}^{2+} \) influx originated mainly from the external milieu as the omission of Na\(^+\) abolishes the MI-induced \( \text{Ca}^{2+} \) increase (Fig. 2). However, MI induces also \( \text{Ca}^{2+} \) release from the internal stores. Although VOCs were also reported to be involved in hypoxia-induced \( \text{Ca}^{2+} \) increase, the major route for \( \text{Ca}^{2+} \) entry in nonexcitable cells is the SOCs (37). In renal epithelial proximal cells both blockers of SOCs (1, 41) and VOCs (1, 41, 47) were shown to
steadily, suggesting that energized mitochondria are responsible for the first phase of the transient \([Ca^{2+}]_i\), increase. It is widely accepted that \([Ca^{2+}]_i\), modification during MI is bound to the level of ATP loss, a link established mainly by the ATP-fueled \(Ca^{2+}\) pumps (4, 28, 39). On the other hand, there is evidence to show that the ATP level is not the only factor involved in \(Ca^{2+}\) regulation during MI (50). Whereas in MDCK cells the transient increase in \([Ca^{2+}]_i\), occurs in the absence of ATP (46), in HCRE cells the ATP level is significant during this process (Fig. 1). This suggests, at least in HCRE cells, that ATP level does not directly determine the first phase of the transient \([Ca^{2+}]_i\), increase. In agreement with this observation, in Jurkat cells, \(Ca^{2+}\) entry via SOCs was shown to be depressed owing to mitochondria uncoupling and not related to the cellular ATP-to-ADP ratio (30). These findings indicate that short-time MI effects may occur through more complicated signaling pathways rather than through the direct inhibition of ATP production. Although similar findings were reported in T lymphocytes (21, 22) as well as in glioma C6, Ehrlich ascites tumor cells, and human fibroblasts (52), where mitochondrial uncouplers inhibited the rate of \(Ca^{2+}\) entry via SOCs, this is the first report showing an association between MI and a possible mitochondrial signaling effect.

**Possible MI-induced signaling events mediated by mitochondria.** Recent evidence points to a dynamic interplay between mitochondria, ER, and the plasma membrane in store-operated \(Ca^{2+}\) entry (15, 22, 32, 36, 37). In this work, the data show that in HCRE cells, energized mitochondria are required to mediate the \(Ca^{2+}\) entry during the first phase of MI, probably via SOCs. Hence it seems that there is a cross talk between mitochondria and the structures responsible for \(Ca^{2+}\) entry. Interestingly, this communication is impaired during the second exposure to MI, when \([Ca^{2+}]_i\), increase is continuous (Fig. 2) and shows similarities with \([Ca^{2+}]_i\), increase in the presence of FCCP (Fig. 4B). In agreement with this observation, it was shown that ATP depletion inhibits the SOC-mediated \(Ca^{2+}\) entry (14, 24, 33). Finally, it is well documented that the mitochondrial activity is severely impaired in proximal tubules subjected to hypoxia-reoxygenation (53, 54), which could be the overall source of the lack of communication.

In endothelial cells it was recently suggested that \(Ca^{2+}\) entering via SOCs transits mitochondria before being delivered to ER and that this has an impact on \(Ca^{2+}\) signaling and \(Ca^{2+}\) homeostasis (31, 37). The present data in this work does not address the role of ER in this particular signaling process directly. However, in the presence of thapsigargin, MI induced only a slight increase in \([Ca^{2+}]_i\), only \(-13\) mM (Fig. 6A). Nevertheless, the starting level was higher (\~2.5 times) than the basal \([Ca^{2+}]_i\). It is possible that the lack of MI-induced \([Ca^{2+}]_i\), increase in the presence of thapsigargin is due either to the impairment of capacitative calcium entry itself (29) or to the suppression of communication between ER and mitochondria. It is worth noting that FCCP depolarizes the mitochondrial membrane and thus releases mitochondrial \(Ca^{2+}\). This process is shown in the Fig. 4B, where FCCP alone induces a short transient increase in \([Ca^{2+}]_i\). Whether mitochondrial \(Ca^{2+}\) depletion is the explanation for the lack of communication still has to be investigated.

What can be the role of energized mitochondria dependent MI in the induced \([Ca^{2+}]_i\), increase? Besides general inhibition of protein synthesis, hypoxia upregulates the genes responsible for the adaptation to this stress (19). This process is mediated by the hypoxia-inducible factors (HIFs). In renal epithelia, HIF-1 is considered the key mediator of acute hypoxic signaling (42, 43). The link between the activation of these factors and the onset of the hypoxic event could be associated with the \([Ca^{2+}]_i\), elevation. In line with this suggestion, a recent report shows that the transcription activation of HIF proteins is dependent on the hypoxia-induced \(Ca^{2+}\) influx (23). On the other hand, mitochondria-dependent \([Ca^{2+}]_i\), elevation could be seen as an event involved in the process of oxygen sensing, in which mitochondria have a dominant role (51).

**Mitochondrial NCX is involved in the \(Ca^{2+}\) clearance during MI.** The recovery phase of the transient \([Ca^{2+}]_i\), increase can be due to the \(Ca^{2+}\) extrusion out of the cells, via ATP-fueled plasma \(Ca^{2+}\) pumps or plasma membrane NCX and/or buffering capacity of internal stores, such as ER and/or mitochondria. Besides being a blocker of SOCs, La\(^{3+}\) is a potent blocker of plasma membrane \(Ca^{2+}\) pumps (5, 6). The recovery of the \(La^{3+}\) sensitive \([Ca^{2+}]_i\), increase is not influenced by \(La^{3+}\), and thus the \(Ca^{2+}\) clearance in this particular condition is unlikely to be mediated by plasma \(Ca^{2+}\) pumps (Fig. 4A). However, the data cannot provide information about the clearance of the \(La^{3+}\) insensitive \([Ca^{2+}]_i\), increase, which could be mediated by these pumps. The activity of plasma membrane \(Na^+\)/\(Ca^{2+}\) exchanger is expected to be diminished during MI, as the consequence of the increase in \([Na^{+}]_i\), (Fig. 5). Finally, ER appears to be not responsible for the calcium clearance seen during MI in HCRE cells (Fig. 6A). As discussed, the ER depletion in \(Ca^{2+}\) disables probably the capacitative \(Ca^{2+}\) entry at the onset of MI. In these conditions, although \(Ca^{2+}\) overload is generated mainly by the ER incapacity to take up \(Ca^{2+}\), the \([Ca^{2+}]_i\), recovery process is still present.

Mitochondria can take up \(Ca^{2+}\) by means of the mitochondrial \(Ca^{2+}\) uniporter and via the mitochondrial NCX. The activity of the \(Ca^{2+}\) uniporter relies on the high \(\Delta \Psi\) maintained across the inner mitochondrial membrane by mitochondrial respiration. When \(\Delta \Psi\) collapses, mitochondrial \(Ca^{2+}\) is released. This is shown in Fig. 4B, where a peak-type response in \([Ca^{2+}]_i\), follows the addition of FCCP. During MI, because of the gradual collapse of \(\Delta \Psi\), the uniporter contribution in the \(Ca^{2+}\) clearance is reduced and less and less important whereas the energetic deficit advances (2, 46, 53). The blockage of mitochondrial NCX does not significantly modify the increase in \([Ca^{2+}]_i\), during MI, but the recovery efficiency was different (Fig. 7). Moreover, like MDCK cells, HCRE cells show a \(Na^+\)-dependent \(Ca^{2+}\) clearance (Fig. 6B). The similarities between \([Ca^{2+}]_i\), increase in the presence and in the absence of CGP-37157 point out that mitochondria are not the source of \(Ca^{2+}\), because the normal route of \(Ca^{2+}\) exit from mitochondria, the mitochondrial NCX, is blocked. In turn, it is likely that mitochondria take up \(Ca^{2+}\) during the recovery phase via mitochondrial NCX working in the entry mode, as showed in MDCK cells (2, 46). According to these reports, the activity of mitochondrial NCX during the second phase of MI-induced transient \(Ca^{2+}\) increase, and thus the \(Ca^{2+}\) buffering capacity, depends on suitable amounts of cytosolic and mitochondrial \(Na^+\). By the use of CoronaRed, a probe for mitochondrial \(Na^+\), it was showed that in a normal \(Na^+\) environment, MI induces a sustained increase in mitochondrial \([Na^+]_i\) which peaks about at the onset of \([Ca^{2+}]_i\), recovery (2). This increase was followed
by a slow decrease, corresponding to the [Ca\(^{2+}\)]\(_i\) recovery period and sustained by the activity of mitochondrial NCX working in reverse mode. In the present study, after 10 min of preincubation in a Na\(^+-\)free medium and when [Na\(^+\)]\(_i\) is decreased to about half of the control, MI induces in HCRE cells a fourfold increase in [Na\(^+\)]\(_i\), in the first 5 min (Fig. 5). Notably, this increase is completely abolished when the mitochondrial NCX is blocked. Thus, in a Na\(^+-\)free medium, MI triggers the activity of the mitochondrial NCX, which exchanges cytosolic Ca\(^{2+}\) for the available mitochondrial Na\(^+\). It is instructive to examine the differences between the two situations. First, whereas in a normal Na\(^+\) environment the MI-associated rise in [Na\(^+\)]\(_i\) induces an increase in mitochondrial Na\(^+\) that will fuel the recovery in [Ca\(^{2+}\)]\(_i\), in a Na\(^+-\)free medium the recovery fails as the consequence of low stores of mitochondrial Na\(^+\). In a Na\(^+-\)free medium, it is possible to consider the rate of [Na\(^+\)]\(_i\) increase and respective decrease connected with the rate of mitochondrial NCX exchange. In this light, during MI, the steady decrease in [Na\(^+\)]\(_i\), after the peak is the result of a continuous decrease in mitochondrial Na\(^+\) concentration (Fig. 5). Secondly, in a Na\(^+-\)free medium, the mitochondrial NCX reverses from the beginning, as the consequence of a favorable Na\(^+\) gradient between the mitochondrial matrix and cytosol. Although it is difficult to draw definitive conclusions about the intimate exchange process, these experiments clearly show that the mitochondrial NCX can reverse during MI.

Taking into account that [Ca\(^{2+}\)]\(_i\) recovery during MI was impaired in HCRE cells in the presence of GP-37157 and abolished in the absence of external Na\(^+\), on the basis of the similarities reported in MDCK cells (2, 46) mitochondrial NCX appears to be one of the factors involved in Ca\(^{2+}\) clearance. This reversal of mitochondrial NCX during hypoxia has also been observed in rat cardiomyocytes (16, 17).

Summary. Similarly to the previous findings in MI challenged MDCK cells (46), in HCRE cells [Ca\(^{2+}\)] shows a transient increase during MI. The first phase of the [Ca\(^{2+}\)] transient increase is dependent on the preservation of the inner mitochondrial potential, it is likely mediated by SOCs, and it seems to represent a mitochondria-mediated signaling event, triggered by the onset of MI. The [Ca\(^{2+}\)] recovery phase partially depends on the activity of mitochondrial NCX, which can reverse during MI. These results show that in human renal epithelial proximal cells subjected to MI mitochondria play an important role in calcium regulation.

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