NCLX Protein, but Not LETM1, Mediates Mitochondrial Ca\(^{2+}\) Extrusion, Thereby Limiting Ca\(^{2+}\)-induced NAD(P)H Production and Modulating Matrix Redox State\(^*\)

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Background: Whether mitochondrial Ca\(^{2+}\) extrusion is mediated by NCLX (mitochondrial sodium/calcium exchanger) or LETM1 (leucine zipper-EF-hand-containing transmembrane protein 1) and controls matrix redox state is unknown.

Results: NCLX, but not LETM1, increases Ca\(^{2+}\) extrusion, limits NAD(P)H production, and promotes matrix oxidation.

Conclusion: NCLX controls the duration of matrix Ca\(^{2+}\) elevations and their impact on redox signaling.

Significance: NCLX is a potential target for the treatment of redox-dependent diseases.

Mitochondria capture and subsequently release Ca\(^{2+}\) ions, thereby sensing and shaping cellular Ca\(^{2+}\) signals. The Ca\(^{2+}\) uniporter MCU mediates Ca\(^{2+}\) uptake, whereas NCLX (mitochondrial Na/Ca exchanger) and LETM1 (leucine zipper-EF-hand-containing transmembrane protein 1) were proposed to exchange Ca\(^{2+}\) against Na\(^{+}\) or H\(^{+}\), respectively. Here we study the role of these ion exchangers in mitochondrial Ca\(^{2+}\) extrusion and in Ca\(^{2+}\)-metabolic coupling. Both NCLX and LETM1 proteins were expressed in HeLa cells mitochondria. The rate of mitochondrial Ca\(^{2+}\) efflux, measured with a genetically encoded indicator during agonist stimulations, increased with the amplitude of mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{mt}\)) elevations. NCLX over-expression enhanced the rates of Ca\(^{2+}\) efflux, whereas increasing LETM1 levels had no impact on Ca\(^{2+}\) extrusion. The fluorescence of the redox-sensitive probe roGFP increased during [Ca\(^{2+}\)]\(_{mt}\) elevations, indicating a net reduction of the matrix. This redox response was abolished by NCLX overexpression and restored by the Na\(^{+}\)/Ca\(^{2+}\) exchanger inhibitor CGP37157. The [Ca\(^{2+}\)]\(_{mt}\) elevations were associated with increases in the auto-fluorescence of NAD(P)H, whose amplitude was strongly reduced by NCLX overexpression, an effect reverted by Na\(^{+}\)/Ca\(^{2+}\) exchange inhibition. We conclude that NCLX, but not LETM1, mediates Ca\(^{2+}\) extrusion from mitochondria. By controlling the duration of matrix Ca\(^{2+}\) elevations, NCLX contributes to the regulation of NAD(P)H production and to the conversion of Ca\(^{2+}\) signals into redox changes.

Ca\(^{2+}\) is a versatile intracellular messenger controlling most cellular processes. In order to maintain normal signaling function, tight spatial/temporal control of Ca\(^{2+}\) is essential. To achieve such tight regulation, cells are equipped with a number of proteins mediating the transport of Ca\(^{2+}\) across the plasma membrane, the endoplasmic reticulum, and the inner mitochondrial membrane (1). mitochondria contribute to the shaping of Ca\(^{2+}\) signals through Ca\(^{2+}\) uptake and release (2–9). At the same time, the associated [Ca\(^{2+}\)]\(_{mt}\) transients act as signals to stimulate energy metabolism.

The amplitude and duration of [Ca\(^{2+}\)]\(_{mt}\) elevations reflect the balance between uptake and release mechanisms (10–13). Uptake is performed by the recently identified mitochondrial Ca\(^{2+}\) uniporter (MCU) (14, 15), whose activity is tightly controlled by the regulatory molecules MICU1, MICU2, MCRY1, and EMRE (16–19). The MCU forms a channel with high selectivity but low affinity for Ca\(^{2+}\) (10, 20). Despite this low affinity, mitochondria can accumulate large amounts of Ca\(^{2+}\) during cell stimulation when exposed to microdomains of high Ca\(^{2+}\) concentration (21), forming in the vicinity of intracellular Ca\(^{2+}\) release or plasma membrane Ca\(^{2+}\) entry channels (22, 23).

Mitochondrial Ca\(^{2+}\) uptake activates several Ca\(^{2+}\)-dependent matrix enzymes that stimulate energy metabolism (24, 25) and ATP synthase-dependent respiration (26). Prolonged (pathological) accumulation of Ca\(^{2+}\) in the matrix space can lead to mitochondrial Ca\(^{2+}\) overload, followed by mitochondrial permeability transition pore opening (27–29), resulting in the activation of cell death signals (30, 31). To avoid this transition from stimulatory to detrimental effects of Ca\(^{2+}\), mitochondria possess two membrane systems to extrude Ca\(^{2+}\); the Na\(^{+}\)/Ca\(^{2+}\) exchanger and the H\(^{+}\)/Ca\(^{2+}\) exchanger (5, 6). Two mitochondrial inner membrane proteins, namely NCLX (32) (sodium/calcium exchanger protein, mitochondrial; or sodium/potassium/calcium exchanger 6, mitochondrial; or solute carrier family 24 member 6) and LETM1 (33) (LETM1 and EF-

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3 The abbreviations used are: [Ca\(^{2+}\)]\(_{mt}\), mitochondrial [Ca\(^{2+}\)]; MCU, mitochondrial Ca\(^{2+}\) uniporter; ROS, reactive oxygen species.
hand domain-containing protein 1, mitochondrial; or leucine zipper-EF-hand-containing transmembrane protein 1) have been recently proposed to exchange Ca²⁺ against Na⁺ or H⁺, respectively. Functional analysis strongly suggests that NCLX is a mitochondrial Na⁺/Ca²⁺ exchanger because overexpression of this protein enhances mitochondrial Ca²⁺ efflux, whereas its knockdown diminishes Ca²⁺ extrusion. Furthermore, pharmacological inhibition of mitochondrial Ca²⁺ efflux with the benzothiazepine derivative CGP37157 completely blocks NCLX-dependent Ca²⁺ export. LETM1 was proposed to be a high affinity mitochondrial Ca²⁺/H⁺ exchanger (33, 34) able to drive both extrusion and uptake of Ca²⁺ into energized mitochondria at submicromolar Ca²⁺ concentrations. Previous studies, however, indicated that LETM1 mediates mitochondrial K⁺/H⁺ exchange (35, 36), and the contribution of LETM1 to mitochondrial Ca²⁺ transport is not yet firmly established (37). One factor hindering studies of mitochondrial Ca²⁺ extrusion is the large variability in the kinetics of mitochondrial Ca²⁺ efflux between cells during physiological stimuli. Perfectly detailed protocols have been available from the 1970s, for the quantitative analysis of Ca²⁺ efflux, and an elegant series of studies carried out by Carafoli and co-workers (38, 39) on isolated mitochondria examined the pathway and mechanism of Ca²⁺ release. Nevertheless, protocols enabling the quantitative analysis of mitochondrial Ca²⁺ efflux in live cells, where the analysis of this process is complicated by cell-to-cell variability, are lacking.

The transient matrix Ca²⁺ elevations have several effects on mitochondrial function. The energetic redox balance in particular is a primary target of the mitochondrial Ca²⁺ homeostasis (40), with strong impact on metabolic regulation (41) and human health (42). Inside the organelle, Ca²⁺ activates oxidative metabolism and respiration. In addition, [Ca²⁺]mt elevations can have several and sometimes opposing effects on the redox balance. On the one hand, [Ca²⁺]mt elevations activate Ca²⁺-dependent dehydrogenases, accelerating NADH production (25). As a result, the ratio of the redox couple NAD(P)H/NAD(P) will increase (43–47). On the other hand, [Ca²⁺]mt elevations accelerate respiration. This will increase the associated formation of reactive oxygen species (ROS) (48, 49), with a net oxidizing effect in the matrix space.

Here we have studied the role of NCLX and LETM1 in the export of Ca²⁺ from the mitochondrial matrix space. In order to properly describe mitochondrial Ca²⁺ export kinetics, we have applied a biparametric single-cell analysis. This novel approach allowed us to determine the contribution of different Ca²⁺ export systems in an amplitude-dependent manner. Furthermore, we have assessed the importance of Ca²⁺ extrusion kinetics in the regulation of oxidative metabolism and in the control of the mitochondrial redox state.

**EXPERIMENTAL PROCEDURES**

Reagents—Histamine, dithiothreitol (DTT), H₂O₂, and rotenone were obtained from Sigma, and CGP37157 was from Calbiochem. Preparation of NCLX-encoding plasmid was described previously (32). The 4mtD3cpv construct (50) was provided by Drs. Amy Palmer and Roger Tsien (University of California, San Diego). The mitochondrial redox indicator roGFP1 (51) was provided by Dr. S. James Remington (University of Oregon). The LETM1-encoding plasmid (35) was provided by Dr. Luca Scarrazza (University of Geneva). The mitochondrial pH sensor mitoSypHer was described previously (52).

**Cell Culture and Transfection**—Minimal essential medium (DMEM), fetal calf serum, penicillin, streptomycin, and Lipofectamine 2000 transfection reagent were from Invitrogen. HeLa cells were cultured in DMEM + 10% fetal calf serum, as described previously (53). For overexpression experiments, cells were plated on 25-mm diameter glass coverslips and co-transfected with the appropriate construct (NCLX, LETM1, or pcDNA3; 1 μg/ml) and a construct encoding a probe for mitochondrial Ca²⁺ (4mD3cpv), redox status (roGFP1), or mitochondrial pH (mitoSypHer) at a 2:1 ratio, using Lipofectamine 2000 transfection reagent. All experiments were performed 2 days after transfection.

**Cell Lysis, mitochondrial Isolation, and Western Blotting**—Whole cells were lysed for 30 min on ice in lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), supplemented with protease inhibitors (Roche Applied Science). The lysate was centrifuged at 14,000 × g for 20 min, and the protein content of the supernatant was determined using a BCA protein assay (Pierce). Mitochondrial fractions were obtained by differential centrifugation (33, 34) able to drive both extrusion and uptake of Ca²⁺ into energized mitochondria at submicromolar Ca²⁺ concentrations. Previous studies, however, indicated that LETM1 mediates mitochondrial K⁺/H⁺ exchange (35, 36), and the contribution of LETM1 to mitochondrial Ca²⁺ transport is not yet firmly established (37). One factor hindering studies of mitochondrial Ca²⁺ extrusion is the large variability in the kinetics of mitochondrial Ca²⁺ efflux between cells during physiological stimuli. Perfectly detailed protocols have been available from the 1970s, for the quantitative analysis of Ca²⁺ efflux, and an elegant series of studies carried out by Carafoli and co-workers (38, 39) on isolated mitochondria examined the pathway and mechanism of Ca²⁺ release. Nevertheless, protocols enabling the quantitative analysis of mitochondrial Ca²⁺ efflux in live cells, where the analysis of this process is complicated by cell-to-cell variability, are lacking.

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Mitochondrial Ca²⁺ Measurements—Experiments were performed in HEPES buffer containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM Hepes, 10 mM glucose, pH 7.4, with NaOH at 37 °C. Glass coverslips were inserted in a thermostatic chamber (Harvard Apparatus, Holliston, MA), and solutions were changed by hand. Cells were imaged on an Axiovert s100 TV using a ×40, 1.3 numeric aperture oil immersion objective (Carl Zeiss AG, Feldbach, Switzerland) and a cooled, 16-bit CCD back-illuminated frame transfer MicroMax camera (Roper Scientific, Trenton, NJ). [Ca²⁺]mt was measured with the genetically encoded 4mtD3cpv sensor. Cells were excited at 430 nm through a 455DRLP dichroic and alternately imaged with 480AF30 and 535DF25 emission filters (Omega Optical). Images were acquired every 2 s. Fluorescence ratios were calculated in MetaFluor 6.3 (Universal Imaging) and analyzed in Excel (Microsoft) and GraphPad Prism 5 (GraphPad). [Ca²⁺]ₘₜ was calculated in situ in semipermeabilized cells as described previously (55) from 4mtD3cpv ratios (R) using the following equation.

\[
[Ca^{2+}] = \frac{K^d \times (R - R_{min})/(R_{max} - R)}{n}
\]

(Eq. 1)

R_{min} was obtained by treating the cells with 1 mM EGTA along with 10 μM ionomycin, and R_{max} was obtained by treating the
cells with 10 μM ionomycin and 10 mM Ca^{2+}. The maximal Ca^{2+} efflux rates were calculated by performing a first order derivative on the data obtained during the first minute of the decay phase of the Ca^{2+} response.

**Mitochondrial Matrix pH Measurements in Permeabilized Cells**—Ratiometric measurements of the mitochondrial pH were performed on the same instrument as for [Ca^{2+}]_{mt} measurements, using the mitochondrial targeted sensor Mito-SypHer. Cells were alternately excited at 420 and 490 nm through a 505DCXR dichroic filter and imaged with a 535DF25 band pass filter (Omega Optical) as described previously (52). Images were acquired every 5 s. MitoSypHer-expressing HeLa cells were permeabilized on the microscope with a 1-min exposure to digitonin (100 mM) in Ca^{2+}-free intracellular buffer, containing 235 mM sucrose, 20 mM HEPES, 5 mM succinic acid, 1 mM EGTA, adjusted to pH 7.4 with N-methyl-D-glucamine. After digitonin washout, cells were kept in intracellular buffer for 10 min, before K^{+}-driven H^{+} efflux was evoked by changing the intracellular solution with a K^{+}-glucosate solution containing 50 mM potassium glucosate, 135 mM sucrose, 20 mM HEPES, 5 mM succinic acid, 1 mM EGTA, adjusted to pH 7.4 with N-methyl-D-glucamine. The ratiometric 490/420 signals were normalized to both the minimum of fluorescence (autofluorescence close to maximal), following the complex I inhibitor rotenone, which results in the accumulation of NAD(P)H (40). The transient amplification of NAD(P)H responses, each experiment was concluded by adding the mitochondrial targeted sensor 4mtD3cpv. Application of the endoplasmic reticulum Ca^{2+}-mobilizing agonist histamine rapidly increased [Ca^{2+}]_{mt} to ~2.5 mM (Fig. 1A), but the amplitude and kinetics of the elevations were highly variable, even between cells of the same clonal population recorded simultaneously (Fig. 1B).

**RESULTS**

**Biparametric Analysis of Mitochondrial Ca^{2+} Extrusion Rates**—To assess the kinetics of Ca^{2+} efflux from mitochondria, we measured [Ca^{2+}]_{mt} changes evoked by physiological agonists in HeLa cells with the mitochondrially targeted Ca^{2+} sensor 4mtD3cpv. Application of the endoplasmic reticulum Ca^{2+}-mobilizing agonist histamine rapidly increased [Ca^{2+}]_{mt} elevations (Fig. 1C). The efflux rates were then expressed as a function of the corresponding signal amplitude by aggregating the data over defined ranges of [Ca^{2+}]_{mt} which allowed us to model the [Ca^{2+}]_{mt}-Ca^{2+} efflux relationship by an exponential fit (Fig. 1D). This approach enabled us to include all of the cells recorded while preserving the complexity of the underlying biological process.

**NCLX Levels but Not LETM1 Levels Modulate Matrix Ca^{2+} Extrusion at High [Ca^{2+}]_{mt}**—We then assessed the contribution of NCLX and LETM1 to mitochondrial Ca^{2+} efflux in HeLa cells (Fig. 2A). NCLX overexpression (32) did not alter the average amplitude of the [Ca^{2+}]_{mt} elevations evoked by histamine (not shown) but accelerated the kinetics of mitochondrial Ca^{2+} efflux (Fig. 2B). The biparametric analysis revealed that NCLX accelerated Ca^{2+} efflux exclusively in cells undergoing large [Ca^{2+}]_{mt} elevations (Fig. 2C). Separate analysis of cells exhibiting small (∆R/∆t ≤ 0.3) and large (∆R/∆t > 0.3) elevations confirmed that NCLX enhanced mitochondrial Ca^{2+} extrusion only in cells experiencing large [Ca^{2+}]_{mt} elevations (Fig. 2D). CGP37157, an inhibitor of the mitochondrial Na^{+}/Ca^{2+} exchanger, almost completely prevented Ca^{2+} efflux, regardless of NCLX overexpression (Fig. 2B–D). The inhibition was reversible (not shown) and particularly evident in cells undergoing large [Ca^{2+}]_{mt} elevations (Fig. 2D). We then tested whether the proposed Ca^{2+}/H^{+} exchanger LETM1 could affect Ca^{2+} efflux, possibly over a range of [Ca^{2+}]_{mt} distinct from NCLX. Overexpression of LETM1 (35) was validated by Western blot, and the function of the overexpressed protein was tested in permeabilized cells (Fig. 3A and B). LETM1 was originally identified as a key element of mitochondrial volume homeostasis through regulation of K^{+}/H^{+} exchange (37). In our hands, overexpression of LETM1 increased K^{+}-driven...
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**FIGURE 1.** Biparametric analysis of mitochondrial Ca\(^{2+}\) extrusion rates. HeLa cells were transiently transfected with the mitochondrial Ca\(^{2+}\)-probe 4mtD3cpv. A, typical [Ca\(^{2+}\)]\(_{\text{mt}}\) response evoked by histamine (50 \(\mu\)M) in HeLa cells. The averaged peak amplitude of 26 cells \((n = 4\) experiments) is 2.5 \(\pm\) 0.79 \(\mu\)M. B, original recordings of three HeLa cells \((i, ii, and iii)\) from the same coverslip stimulated with 100 \(\mu\)M histamine. C, measurements of maximal mitochondrial Ca\(^{2+}\) efflux rates \((\Delta R/s)\) and [Ca\(^{2+}\)]\(_{\text{mt}}\) signal amplitude \((\Delta R/R_0)\). D, Ca\(^{2+}\) efflux rates as a function of the [Ca\(^{2+}\)]\(_{\text{mt}}\) signal amplitude. Data from 64 cells \((n = 10)\) were aggregated for different ranges of [Ca\(^{2+}\)]\(_{\text{mt}}\) values and expressed as a function of [Ca\(^{2+}\)]\(_{\text{mt}}\). The horizontal error bars are the mean \(\pm\) S.E. (error bars) of the Ca\(^{2+}\) signal amplitude. A dotted line shows the exponential regression through the data.

matrix proton extrusion (Fig. 3B), consistent with the overexpression of a functional K\(^{+}/H^{+}\) exchanger (Fig. 3B). On the other hand, LETM1 did not alter Ca\(^{2+}\) efflux rates, regardless of the amplitude of [Ca\(^{2+}\)]\(_{\text{mt}}\) elevations (Fig. 3, C–E). These data demonstrate that NCLX but not LETM1 levels limit the rates of Ca\(^{2+}\) extrusion from mitochondria, an effect most apparent during large [Ca\(^{2+}\)]\(_{\text{mt}}\) elevations in HeLa cells that endogenously express both exchangers.

**NCLX Levels Modulate the Mitochondrial Redox State during Stimulation**—Through its impact on the duration of [Ca\(^{2+}\)]\(_{\text{mt}}\) elevations, NCLX may affect intramitochondrial Ca\(^{2+}\)-dependent processes and alter the mitochondrial redox status. To test this possibility, we measured the mitochondrial redox state using the genetically encoded redox-sensitive probe roGFP1 (51), which contains engineered surface cysteine groups positioned to reversibly form disulfide bonds. Expression of matrix-targeted roGFP1 labeled mitochondria (Fig. 4A), and the fluorescence signal increased during histamine application, indicating a more reduced state (Fig. 4B). To compare redox responses, we determined the full signal range of the probe in each experiment using peroxide to oxidize roGFP1 followed by reduction of the probe with dithiothreitol (Fig. 4B). NCLX overexpression did not affect the basal redox state prior to histamine addition (Fig. 4C) but completely prevented the histamine-induced redox changes (Fig. 4, B and D), an effect that was partially reversed by CGP37157 (Fig. 4, B and D). These results demonstrate that NCLX levels regulate the mitochondrial redox state, probably by altering the duration of [Ca\(^{2+}\)]\(_{\text{mt}}\) elevations.

**NCLX Levels Limit Histamine-induced Mitochondrial NAD(P)H Production**—The observed net reduction of mitochondrial matrix redox state led us to speculate that NCLX, via its effect on matrix Ca\(^{2+}\), might regulate Ca\(^{2+}\)-dependent matrix dehydrogenases. To test this possibility, we measured changes in NAD(P)H autofluorescence by two-photon microscopy. Histamine application increased the NAD(P)H autofluorescence of HeLa cells (Fig. 5A), indicating a net reduction of NAD(P)\(^+\) to NAD(P)H, confirming earlier studies (56, 57). To compare NAD(P)H responses, we calibrated each recording by adding the complex I inhibitor rotenone to promote maximal accumulation of NAD(P)H, followed by peroxide to decrease the autofluorescence to the minimal value. NCLX overexpression did not affect the basal autofluorescence levels of HeLa cells (Fig. 5B) but severely blunted histamine-induced NAD(P)H formation, by 73%, an effect fully prevented by CGP37157 (Fig. 5C). We conclude that NCLX levels are critical for the [Ca\(^{2+}\)]\(_{\text{mt}}\)-dependent regulation of the mitochondrial oxidative metabolism.

**DISCUSSION**

Mitochondria sense and shape Ca\(^{2+}\) signals during cell stimulation (2–6, 58) via their ability to take up and subsequently release Ca\(^{2+}\) ions. Ca\(^{2+}\) sequestration in the mitochondrial matrix contributes to the buffering of cytosolic Ca\(^{2+}\) elevations and serves as a signal that activates mitochondrial Ca\(^{2+}\)–dependent processes (24, 25). In contrast, prolonged accumulation of Ca\(^{2+}\) in the matrix triggers mitochondria-induced cell death (28, 31, 58). Here, we assessed the contribution of the two ion exchangers NCLX and LETM1 in the kinetics of mitochondrial Ca\(^{2+}\) extrusion and in the control of the matrix redox state.

The proteins that transport Ca\(^{2+}\) across the mitochondrial inner membrane were recently identified, and most efforts are currently devoted to defining the mechanism of mitochondrial Ca\(^{2+}\) uptake (14, 15, 21–23, 59, 60). However, the extrusion process is equally critical to achieve mitochondrial Ca\(^{2+}\)-dependent signaling without triggering cell death. Here, we demonstrate that the rates of mitochondrial Ca\(^{2+}\) extrusion are related to the [Ca\(^{2+}\)]\(_{\text{mt}}\) amplitude, with high rates of Ca\(^{2+}\) efflux following large elevations and very slow rates following small elevations. This amplitude-dependent control of mitochondrial Ca\(^{2+}\) export probably serves to maintain [Ca\(^{2+}\)]\(_{\text{mt}}\) in
a range that is sufficient to activate mitochondrial metabolism (24–26) without reaching the levels that could initiate apoptosis. Our observation that Ca\(^{2+}\) extrusion is minimal at low [Ca\(^{2+}\)]\(_{\text{mit}}\) and maximal when mitochondria experience Ca\(^{2+}\) signals of large amplitude therefore suggests that the Ca\(^{2+}\) extrusion system is tuned to avoid long lasting [Ca\(^{2+}\)]\(_{\text{mit}}\) elevations that can trigger cell death by promoting Ca\(^{2+}\)-dependent mitochondrial permeability transition pore opening (28, 31). The nature of this [Ca\(^{2+}\)]\(_{\text{mit}}\)-sensing mechanism is not known, but several studies suggest the existence of regulatory mechanisms controlling mitochondrial Ca\(^{2+}\) export kinetics via direct or indirect interactions with the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger. The protein kinases PKC (61) and PINK1 (62) were reported to modulate the activity of this ion exchanger, and the stomatin-like protein SLP-2, which localizes to the inner mitochondrial membrane, was shown to inhibit mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchange (63). Direct regulation of the exchanger by Ca\(^{2+}\) cannot be excluded, but NCLX does not share the hallmark Ca\(^{2+}\) regulatory site of plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchange proteins (64).

The [Ca\(^{2+}\)]\(_{\text{mit}}\) dependence of the Ca\(^{2+}\) extrusion system, combined with the large cellular variability in the amplitude of [Ca\(^{2+}\)]\(_{\text{mit}}\) responses, prompted us to express Ca\(^{2+}\) efflux rates as a function of the [Ca\(^{2+}\)]\(_{\text{mit}}\) signal amplitude measured in control (white) and NCLX-overexpressing cells without (black) or with 10 \(\mu\)M CGP37157 (blue). Dotted lines, exponential regression through the data. \(\Delta R/R_0\), mitochondrial Ca\(^{2+}\) extrusion signal amplitude. Data are mean ± S.E. (error bars) of 64 (n = 10), 68 (n = 16), and 28 cells (n = 3) for control (white), NCLX (black), and NCLX + CGP37157 (blue). **, \(p < 0.01\); ***, \(p < 0.001\); NS, not significant.

Given the strong impact of NCLX on mitochondrial Ca\(^{2+}\) efflux kinetics, we further investigated its role in the regulation of mitochondrial oxidative metabolism and redox state.
A complex relationship exists between \([\text{Ca}^{2+}]_{\text{mt}}\) and matrix redox processes. \([\text{Ca}^{2+}]_{\text{mt}}\) is able to affect the redox state by activating oxidative metabolism but also by influencing the formation of reactive oxygen species. A rise in \([\text{Ca}^{2+}]_{\text{mt}}\) stimulates several matrix dehydrogenases, which in the presence of substrate are able to increase the NAD(P)H/NAD(P) ratio and, as a consequence, cause a net reduction of the matrix redox state. At the same time as respiration is accelerated, more reactive oxygen species are formed, which should shift redox couples in the direction of oxidation (43–49). Following stimulation with histamine, we observed a net reduction of the redox-sensitive thiol groups of roGFP1 expressed in the mitochondria of HeLa cells (Fig. 4). Similarly, the histamine-induced \([\text{Ca}^{2+}]_{\text{mt}}\) rise also increased the NAD(P)H/NAD(P) ratio (Fig. 5). The net redox changes due to histamine-induced \([\text{Ca}^{2+}]_{\text{mt}}\) elevations appear to be dominated by the activation of \([\text{Ca}^{2+}]_{\text{mt}}\)-dependent dehydrogenases. Such redox changes are known to have further downstream effects modulating electron transport, ATP-synthase (66), and matrix enzyme activities (67). Mitochondrial redox signaling has therefore been proposed to be crucial for the regulation of energy metabolism (41, 68). Furthermore, mitochondrial activation and redox changes are linked to the production of ROS at concentrations that impact signaling functions, as shown for glucose-induced insulin secretion (69). Our results establish a direct link between NCLX-mediated mitochondrial \([\text{Ca}^{2+}]_{\text{mt}}\) efflux and matrix redox state. Redox-dependent processes are therefore sensitive to the regulation of NCLX during stimulus-induced \([\text{Ca}^{2+}]_{\text{mt}}\) elevations. Given that NCLX shortens the duration of the mitochondrial \([\text{Ca}^{2+}]_{\text{mt}}\) transient (Fig. 2, B and C) without significantly lowering the amplitude, the effect on the mitochondrial redox state is surprisingly strong (Fig. 4). These results suggest that the fast uptake of \([\text{Ca}^{2+}]_{\text{mt}}\) is not sufficient to modulate the mitochondrial redox state. Instead, \([\text{Ca}^{2+}]_{\text{mt}}\) elevations must last for a sufficient time to boost NAD(P)H production. This is consistent with previous studies showing that the metabolic decoding of cytosolic \([\text{Ca}^{2+}]_{\text{mt}}\) elevations requires the integration of multiple repetitive elevations (56, 57, 70).

The inhibitor CGP37157 rescued all of the mitochondrial functions affected by NCLX overexpression, indicating that Na+ \(/\text{Ca}^{2+}\) exchange activity accounts for the changes in oxidative metabolism and redox state. In the presence of the inhibitor, \([\text{Ca}^{2+}]_{\text{mt}}\) extrusion was minimal regardless of NCLX overexpression, whereas
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