NCLX is an essential component of mitochondrial Na^+/Ca^{2+} exchange

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Mitochondrial Ca^{2+} efflux is linked to numerous cellular activities and pathophysiological processes. Although it is established that an Na^+-dependent mechanism mediates mitochondrial Ca^{2+} efflux, the molecular identity of this transporter has remained elusive. Here we show that the Na^+/Ca^{2+} exchanger NCLX is enriched in mitochondria, where it is localized to the cristae. Employing Ca^{2+} and Na^+ fluorescent imaging, we demonstrate that mitochondrial Na^+-dependent Ca^{2+} efflux is enhanced upon overexpression of NCLX, is reduced by silencing of NCLX expression by siRNA, and is fully rescued by the concomitant expression of heterologous NCLX. NCLX-mediated mitochondrial Ca^{2+} transport was inhibited, moreover, by CGP-37157 and exhibited Li^+ dependence, both hallmarks of mitochondrial Na^+-dependent Ca^{2+} efflux. Finally, NCLX-mediated mitochondrial Ca^{2+} exchange is blocked in cells expressing a catalytically inactive NCLX mutant. Taken together, our results converge to the conclusion that NCLX is the long-sought mitochondrial Na^+/Ca^{2+} exchanger.

mitochondrial calcium exchanger | mitochondrial calcium homeostasis | sodium calcium exchanger | CGP-37157

Apart from their metabolic role, mitochondria are a major hub of cellular Ca^{2+} homeostasis (1). Powered by the steep mitochondrial membrane potential, Ca^{2+} enters into this organelle via a mitochondrial uniporter and is extruded by either an H^+- or an Na^+-coupled mitochondrial exchanger. Activity of the Na^+/Ca^{2+} exchanger is ubiquitously found in most cell types and tissues studied so far and is particularly robust in excitable cells, whereas the activity of the H^+/Ca^{2+} exchanger is primarily found in nonexcitable cells. Recently a mitochondrial H^+/Ca^{2+} exchanger termed Letm1 has been identified, but its role in Ca^{2+} extrusion is not clear (2). By catalyzing Na^+-dependent Ca^{2+} efflux, the putative mitochondrial Na^+/Ca^{2+} exchanger plays a fundamental role in regulating mitochondrial Ca^{2+} homeostasis (3), oxidative phosphorylation (4), and Ca^{2+} crosstalk among mitochondria, cytoplasm, and the endoplasmic reticulum (ER) (5). Although the activity of this transporter was documented more than 30 years ago (6), its molecular identity remained unknown. We and others previously identified and characterized NCLX, a novel Na^+/Ca^{2+} exchanger, and the single mammalian member of a phylogenetically ancestral branch of the Na^+/Ca^{2+} exchanger superfamily (7, 8). Intriguingly, NCLX catalyzes Na^+- or Li^+-dependent Ca^{2+} transport at similar rates, a feature shared only with the unidentified mitochondrial exchanger. Although the activity of ectopically expressed human NCLX was first monitored at the plasma membrane, the unique phylogenetic and functional properties of NCLX prompted us to investigate whether NCLX is linked to the mitochondrial exchanger.

Results

We initially sought to determine if NCLX is found in the mitochondria. Comparison of NCLX levels in total versus mitochondrial-enriched fractions from mouse heart and brain showed that the 50- and 70-kDa forms of NCLX (7) and an additional 100 kDa form were enriched in the mitochondrial fraction (Fig. 1*A*). Augmentation of NCLX expression was also observed in the mitochondrial fraction from HEK-293 cells heterologously or endogenously expressing NCLX (Fig. 1 *B* and *D* and Figs. S1*A* and S2 *C–F*). Based on the well documented ability of NCX and other membrane proteins to form SDS-resistant dimers, we reasoned that the 100-kDa form is related to such an NCLX dimer. This was confirmed by the simultaneous reduction in expression of the 50-kDa and 100-kDa forms of NCLX in cells transfected with siNCLX (Fig. 1*C*), and by coimmunoprecipitation of NCLX monomers (see Fig. S1*C*). Also, dissociation of the 100-kDa form to the 50-kDa form was observed following lengthy incubation in denaturing buffer (Fig. S1*B*). Intriguingly, the mitochondrial NCLX is remarkably similar in size to molecularly unidentified mitochondrial polypeptides that, when purified and reconstituted, exhibited Na^+/Ca^{2+} exchange activity (10, 11).

We next determined the subcellular distribution of NCLX by analyzing endogenous NCLX expression in plasma membrane, ER, and mitochondrial fractions from HEK-293 cells or rat heart (Fig. 1 *D* and *E*) and found that endogenous NCLX was enriched primarily in the mitochondrial fraction, but not in the ER or the plasma membrane, as previously suggested (7, 9). Altogether, these results indicate that the mitochondria are the major cellular site of NCLX localization.

Additional support for the mitochondrial localization of NCLX came from immunoelectron microscopy analysis of brain sections and CHO cells. Intense labeling was observed in mitochondria, but not in nuclear, sarcoplasmic reticulum, or plasma membrane structures or in preparations reacted with preimmune serum (Fig. 2 *A–C*). Finally, to define the localization of NCLX within mitochondria, a postembedding cryo-EM procedure featuring protein A gold–labeled, anti-NCLX antibodies was employed (Fig. 2 *D–E* and Fig. S1*D*). Discrete gold particles were observed within mitochondria, particularly within the cristae, of control or NCLX-overexpressing SHSY-5Y cells. Individual gold particles unrelated to mitochondria were occasionally observed (see *Materials and methods*), showed no organelle or compartment preference, and apparently represent nonspecific background staining. Comparison of the number of gold particles observed on ultrathin mitochondrial profiles from control and

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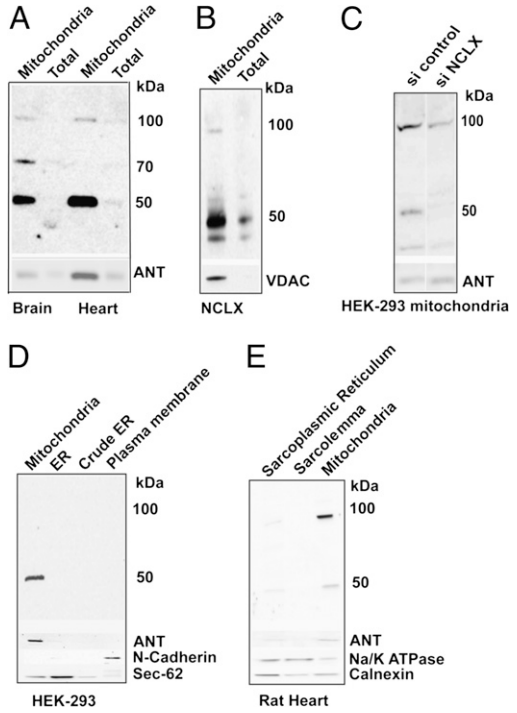

Fig. 1. NCLX is localized to mitochondria. (*A*) Immunoblot analysis of NCLX in total tissue lysate (*Total*) and mitochondrial fractions (*Mitochondria*) purified from mouse heart and brain (15 μg). (*B*) Immunoblot analysis of total cellular and mitochondrial fractions purified from HEK-293-T cells overexpressing murine NCLX (10 μg). (*Lower*) Immunoblots of ANT or VDAC serving as markers. (*C*) Immunoblot analysis of NCLX in mitochondrial fractions purified from HEK-293 cells transfected with siNCLX or scrambled siRNA (*siControl*). Note that siNCLX diminishes expression levels of both the 50-kDa and 100-kDa forms of NCLX. (*D* and *E*) Expression of NCLX in cellular and tissue fractions of the indicated components purified from HEK-293 cells (20 μg) (*D*) or rat heart (10 μg) (*E*). (*Lower*) Immunoblots of ANT (mitochondrial marker), Na^+/K^+ ATPase, or N-cadherin (plasma membrane, *PM*, marker) and Calnexin or Sec-62 (*ER*, marker). Note that the mitochondria are the major site of NCLX localization (the slight NCLX signal in cardiac sarcoplasmic reticulum is presumably related to cross-contamination with mitochondria; see ANT staining).

NCLX-overexpressing SHSY-5Y cells revealed that mitochondria from the latter contained more particles than did control mitochondria (35 vs. 14; $P < 0.01$; Fig. 2*F*). This result indicates that the observed labeling is related to NCLX and that both overexpressed and endogenous NCLX, like the mitochondrial exchanger, are localized to the mitochondrial cristae.

If NCLX is to play a functional role in mitochondria, it should be linked to mitochondrial Ca^{2+} efflux. Mitochondrial Ca^{2+} signals were determined by using the mitochondrial-targeted calcium-sensing protein Pericam [RP-mt (12), also see Fig. S3*B*]. RP-mt was excited at the pH-insensitive 430-nm wavelength and not at the 485-/430-nm ratiometric mode because of the pH sensitivity of Pericam to excitation at the 485-nm wavelength and the potential interference by mitochondrial pH changes we and others have observed (see Fig. S3 *E–G* and ref. 13). In this set of experiments, we have expressed the murine isoform of NCLX that, consistent with previous studies (9), did not reach the plasma membrane and did not exhibit plasma membrane Na^+/Ca^{2+} exchange activity (see Fig. S2 *B* and *F*). Plasma membrane Na^+/Ca^{2+} exchange activity was also absent in the control nontransfected cells (see Fig. S2*B*). These results indicate that neither the endogenous nor the ectopically expressed murine NCLX contribute to plasma membrane Ca^{2+} fluxes. We then asked if overexpression of NCLX will modulate mitochondrial Ca^{2+} efflux. Rates of mitochondrial Ca^{2+} efflux in control cells were taken as the reference point (100%) and changes in activity following NCLX overexpression, silencing, or inhibition were presented as percentage of control. Application of ATP (40 μM) was followed by mitochondrial Ca^{2+} uptake and a subsequent slower Ca^{2+} efflux phase (Fig. 3*A*), corresponding to the established activity of the mitochondrial Ca^{2+} uniporter and the Na^+/Ca^{2+} exchanger, respectively. Mitochondrial Ca^{2+} uptake was similar in NCLX-expressing or control cells but the rate of Ca^{2+} efflux was higher in the NCLX-expressing cells (194 ± 23% of control; Fig. 3 *A* and *C*), indicating that NCLX expression is linked to enhanced mitochondrial Ca^{2+} efflux. Application of ATP triggered a similar increase in cytoplasmic Ca^{2+} (Fig. S4*E*) in the NCLX-overexpressing cells and control. Furthermore, steady-state mitochondrial Ca^{2+} levels and membrane potential were similar in NCLX-expressing and control cells (Fig. S3*A* and Fig. S4 *A–C*), arguing against an indirect effect of NCLX activity on the cytoplasmic Ca^{2+} response or mitochondrial Ca^{2+} uptake. However, when ATP was applied for longer intervals in the presence of Ca^{2+}-containing Ringer solution, thereby imposing a stronger mitochondrial Ca^{2+} load, the cytoplasmic Ca^{2+} levels were elevated in cells overexpressing NCLX (Fig. S4 *F* and *G*). This is consistent with the physiological role of the mitochondrial exchanger in also modulating cytoplasmic Ca^{2+} responses by enhancing mitochondrial Ca^{2+} efflux and augmenting the store-operated calcium entry into cells (13).

Because of the dimeric nature of NCLX (Fig. S1 *B* and *C* and ref. 14), we hypothesized that a catalytically inactive mutant of NCLX would exert a dominant-negative effect on mitochondrial Ca^{2+} efflux, thereby providing an additional and highly specific tool for analyzing the mitochondrial role of NCLX. Accordingly, we generated a mutant in which threonine 468, which is catalytically essential for the activity of NCLX and other members of NCX family, was replaced by a serine (NCLX-S468T) (14). Like the native protein, NCLX-S468T was targeted to the mitochondria (see Fig. S2*E*). Applying the same paradigm as described in Fig. 3*A*, we found that expression of NCLX-S468T was followed by profound inhibition of endogenous mitochondrial Ca^{2+} efflux rate in SHSY-5Y (20 ± 15% of control; Fig. 3 *A* and *C*) or HEK-293 cells (26 ± 5% of control; Fig. S5 *A–B*). These results indicate that NCLX is catalytically essential for mitochondrial Ca^{2+} efflux.

The benzothiazepine compound CGP-37157 is an established inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger (15). We reasoned that, if NCLX is this exchanger, its activity should be blocked by this compound. Application of CGP-37157 (10 μM) to SHSY-5Y cells heterologously expressing NCLX or to control cells resulted in profound inhibition of mitochondrial Ca^{2+} efflux in both (Fig. 3 *B* and *C*). In addition, dose-dependence analysis of the effect of CGP-37157 on Ca^{2+} efflux in NCLX-overexpressing SHSY-5Y cells showed approximately 50% inhibition using 5 μM and complete inhibition of mitochondrial Ca^{2+} efflux at 7.5 μM (Fig. 3*D*). This analysis argues against any heterogeneous effect of CGP-37157 on distinct targeting sites and thus indicates that endogenous and overexpressed NCLX share similar sensitivity to GCP-37157. Moreover, the concentration of CGP-37157 required for inhibition of Ca^{2+} efflux in NCLX-overexpressing SHSY-5Y cells was similar to that reported for inhibition of the mitochondrial exchanger in intact cells (16), and is considerably lower than that reported for partial inhibition of other members of the NCX family (17, 18). Hence, the results of this set of experiments point to NCLX as being the molecular moiety catalytically linked to mitochondrial Ca^{2+} efflux, and its sensitivity to CGP-37157 provides additional evidence that NCLX is the mitochondrial Na^+/Ca^{2+} exchanger.

If NCLX mediates mitochondrial exchange activity, then reducing its endogenous expression should attenuate mitochondrial Ca^{2+} efflux. Transfection of cells with NCLX siRNA (siNCLX) lowered NCLX expression (Fig. 1*C*). These cells exhibit similar resting mitochondrial Ca^{2+} levels as untreated cells, their mitochondria being slightly hyperpolarized (Figs. S3*A* and S4*C*). Then the same ATP-induced mitochondrial Ca^{2+} efflux assay described in Fig. 3*A* was applied. Whereas mitochondrial Ca^{2+}

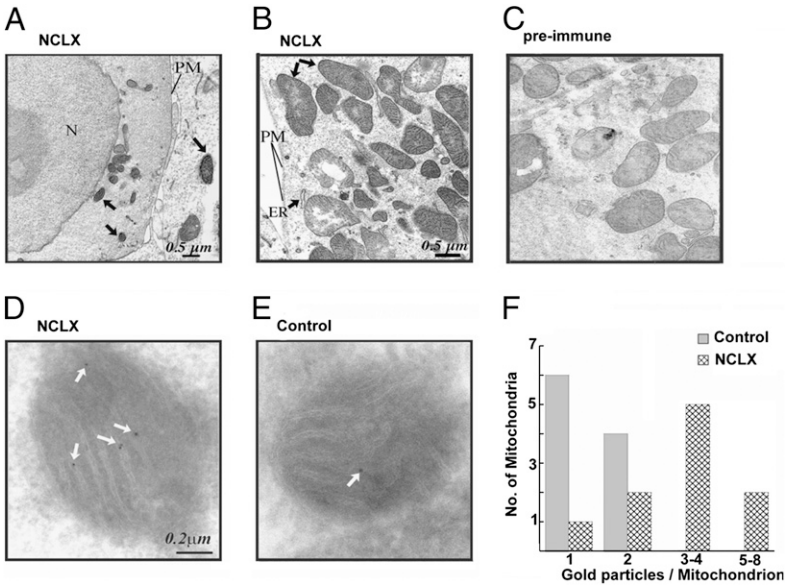

Fig. 2. NCLX is found on the inner membrane of mitochondria. (*A–C*) Electron micrographs of rat cortical slices (*A*) or CHO cells (*B*) stained with anti-NCLX antibodies or NCLX preimmune serum (*C*). *N*, *ER*, and *PM* denote the nucleus, endoplasmic reticulum, and plasma membrane structures, respectively. Positive immunolabeling, defined as the presence of dense DAB precipitate, is observed primarily in the mitochondria. (Scale bar, 0.5 μm.) (*D* and *E*) Immunogold labeling of SHSY-5Y cells overexpressing (*D*) or endogenously expressing (*E*) NCLX. Note that NCLX labeling is found primarily in the cristae of the mitochondria. (Scale bar, 0.2 μm.) (*F*) The distribution of numbers of gold particles per mitochondrion is shown. Quantitative analysis of mitochondrial gold particle distribution shows that the overall number of particles in NCLX-overexpressing cells was 35 versus 14 in the control cells (number of mitochondria examined, $n = 10$ for each group; $^{**}P < 0.01$).

uptake was unaffected, in cells in which NCLX expression had been silenced, the Ca^{2+} efflux rate was reduced by approximately 75% of the rate measured in control cells (22 ± 6.5% of control; Fig. 4*A*). A similar decrease in mitochondrial Ca^{2+} efflux following silencing of NCLX expression was also observed in CHO cells (Fig. S5 *D–F*). We further reasoned that, if NCLX directly participates in endogenous mitochondrial exchange activity, expression of a heterologous species of NCLX should rescue the activity reduced by the silencing. To address this hypothesis, SHSY-5Y cells were transfected with a plasmid encoding NCLX shRNA (see *Materials and Methods*), thereby generating a sequence that specifically targets a UTR region in the human NCLX mRNA sequence (i.e., shNCLX) but not in the murine homologue. Human SHSY-5Y cells transfected with the shNCLX exhibited a 56 ± 6.7% reduction in mitochondrial Ca^{2+} efflux activity compared with control cells (Fig. 4*B*). In contrast, coexpression of murine NCLX with the shNCLX plasmid induced a fourfold increase in the rate of Ca^{2+} efflux versus cells transfected with shNCLX only. Hence, Ca^{2+} efflux activity had been fully restored (Fig. 4*B*). Taken together, the results from this set of experiments support the concept that NCLX is an essential mediator of endogenous mitochondrial Ca^{2+} efflux and that heterologous expression of NCLX is sufficient to functionally complement the loss of the endogenous activity.

We next examined whether NCLX activity is linked to mitochondrial Na^+/Ca^{2+} exchange by assessing whether it displays a strict Na^+ dependence in mediating Ca^{2+} efflux. The Na^+ dependence of mitochondrial NCLX was assayed in digitonin-permeabilized cells, thus allowing equilibration of the intracellular and extracellular compartments and control of the extramitochondrial ionic environment while eliminating any possible interference by plasma membrane transporters. To ascertain that the cells were permeabilized using this protocol, they were loaded with carboxyfluorescein diacetate (CFDA) succinimidyl ester dye that was trapped within the cells. Subsequent addition of digitonin triggered a strong decrease of CFDA fluorescence, suggesting that the entrapped dye was removed out of the cells and hence that the cells were permeabilized (Fig. S3*C*). Permeabilized HEK-293 cells transiently expressing RP-mt alone or together with NCLX were s\perfused with a Ca^{2+}-containing (60 μM) Na^+-free (replaced with $NMDG^+$, see *Materials and Methods*) solution that triggered a similar increase in mitochondrial Ca^{2+} levels in control and NCLX-transfected cells (Fig. S3*D*). Strong Ca^{2+} efflux was monitored only after 20 mM Na^+ (Fig. 5*A*) were reintroduced, indicating that the Ca^{2+} efflux mediated by Na^+-dependent exchange is the major mitochondrial Ca^{2+} extrusion pathway in these cells. A marked increase (259 ± 45%) in mitochondrial Na^+-dependent Ca^{2+} efflux was monitored in NCLX-overexpressing versus control cells (Fig. 5*A*). To directly determine if Na^+ was the counter ion transported into mitochondria by NCLX, the same paradigm was applied to cells overexpressing NCLX loaded with the mitochondrial Na^+-sensitive dye CoroNa Red. As shown in Fig. 5*B*, application of 20 mM Na^+ following mitochondrial Ca^{2+} loading triggered a rise in CoroNa Red fluorescence. The rate of Na^+ influx was increased (170 ± 47%) in NCLX-overexpressing cells compared with controls, but not in cells superfused with Na^+-free solution. indicating that the fluorescence increase resulted from Na^+ influx into the mitochondria. This apparent coupling between mitochondrial Na^+ influx and Ca^{2+} efflux with elevated expression levels of NCLX is consistent with mitochondrial Na^+/Ca^{2+} exchange activity being mediated by this protein. Further substantiating the link between NCLX and mitochondrial exchanger activity, the observed Na^+-dependent Ca^{2+} efflux was profoundly inhibited (to 28 ± 8.7% of control) in HEK-293 cells transfected with the siNCLX construct (Fig. 5*C*). Importantly, Ca^{2+} efflux was virtually eliminated in NCLX-overexpressing (Fig. 5 *A* and *B*) or control (Fig. 5*C*) cells that were superfused with Na^+-free solution. indicating that Ca^{2+} efflux mediated by the mitochondrial exchanger and mitochondrial NCLX share the same strict Na^+ dependence.

Finally, Li^+/Ca^{2+} exchange activity is a unique property of both NCLX (7) and the mitochondrial exchanger (19). We therefore asked if NCLX conducts mitochondrial Li^+-dependent Ca^{2+} transport. Superfusion of permeabilized HEK-293 control cells with 30 mM Li^+ in a Na^+-free solution facilitated robust Ca^{2+} efflux from the mitochondria, consistent with the unique ability of the mitochondrial exchanger to mediate Li^+-dependent Ca^{2+} exchange (Fig. 5*D*). Importantly, silencing of NCLX expression was followed by a strong reduction in the rate of Li^+-dependent Ca^{2+} efflux, (35 ± 8% of control; Fig. 5*D*). Similar attenuation of Li^+-dependent Ca^{2+} efflux activity upon silencing of NCLX expression was also recorded in CHO cells (see Fig. S5 *G–I*). These results indicate that NCLX is the molecular determinant essential for mitochondrial Na^+/Ca^{2+} and Li^+/Ca^{2+} exchange activity.

Discussion

Numerous studies have reported that the Na^+-dependent mitochondrial exchanger, similar to other members of the NCX

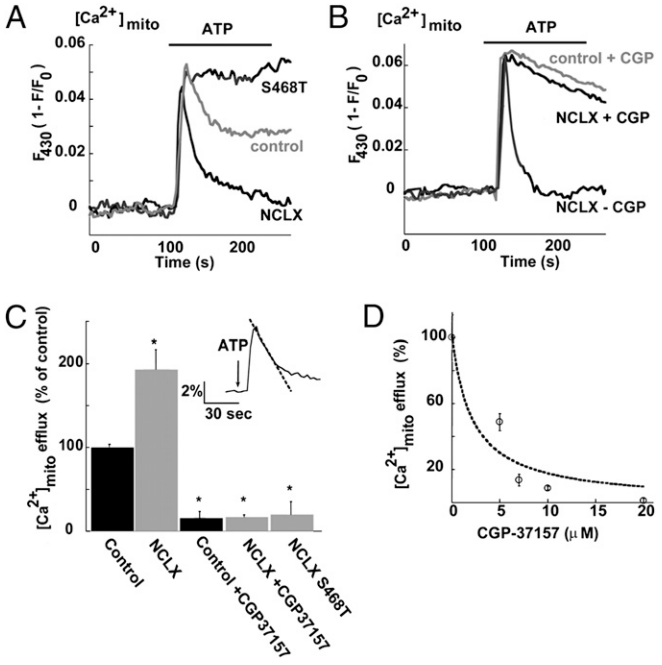

Fig. 3. Expression of NCLX enhances mitochondrial Ca^{2+} efflux that is blocked by mutation in the catalytic site of NCLX and by the mitochondrial exchanger inhibitor CGP-37157. (*A*) SHSY-5Y cells transfected with plasmids encoding the mouse NCLX, NCLX-S468T mutant, or the vector (control) were cotransfected to express RP-mt. Cells were superfused with ATP-containing Ringer solution (40 μM at the indicated time) while monitoring mitochondrial Ca^{2+} fluorescence. Note that enhancement of NCLX levels increased mitochondrial Ca^{2+} efflux, and the mutant was not only inactive but also exerted a dominant-negative effect on endogenous activity. (*B*) Illustration of the same experimental paradigm as in *A* repeated in control or NCLX-expressing cells in the presence of the mitochondrial exchanger inhibitor CGP-37157 (10 μM). Similar inhibition of Ca^{2+} efflux is seen in both. (*C*) Averaged mitochondrial Ca^{2+} efflux rates ($n = 9$; $^{**}P < 0.01$). (*Inset*) Experimental paradigm for Ca^{2+} efflux rate measurement based on determination of the initial rate of the mitochondrial Ca^{2+} efflux phase. (*D*) Dose-dependence analysis of the effect of CGP-37157 on mitochondrial Ca^{2+} efflux in cells expressing NCLX. Mitochondrial Ca^{2+} efflux rate was measured in the presence of the indicated concentrations of CGP-37157 and presented as percentage of the rate measured in its absence. The dashed line is a fit of the data to the equation $I = I_o / [1^+([CGP] / IC_{50})]$.

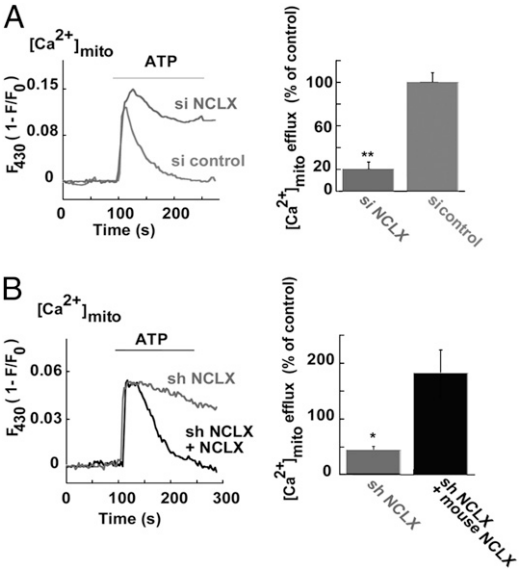

Fig. 4. Silencing the expression of endogenous NCLX decreases mitochondrial Ca^{2+} efflux that can be rescued by expression of recombinant murine NCLX. (*A*) Mitochondrial Ca^{2+} responses following application of ATP (40 μM, as in Fig. 3*A*) was measured in HEK-293 cells cotransfected with either the siNCLX or a scrambled siRNA construct (*si control*) and the RP-mt–expressing plasmid. Averaged rates of mitochondrial Ca^{2+} efflux are shown (*Right*) ($n = 9$, $^{**}P < 0.01$). (*B*) The same experiment as in *A* was performed on SHSY-5Y cells transfected with NCLX shRNA plasmid alone or together with the murine NCLX-encoding plasmid (which is insensitive to the NCLX shRNA construct). Averaged rates of mitochondrial Ca^{2+} efflux are shown (*Right*) ($n = 11$, $^{*}P < 0.05$).

family, mediates Na^+/Ca^{2+} exchange, albeit with unique functional properties (15, 19), including altered selectivity to monovalent cations and distinct sensitivity to inhibitors. In our studies, we have focused on NCLX because, although it is a member of the NCX family, it is phylogenetically and functionally distinct from other known NCX or NCKX family members (20). Support for NCLX being the mitochondrial Na^+/Ca^{2+} exchanger come from the following: (*i*) NCLX is expressed in mitochondria, where it is localized to the inner membrane of this organelle, and has a molecular weight similar to that of the purified mitochondrial exchanger (21). (*ii*) NCLX expression accelerates mitochondrial Ca^{2+} efflux activity, whereas silencing of NCLX expression attenuates this process. Importantly, Ca^{2+} efflux was fully rescued in NCLX-silenced cells by concomitant overexpression of NCLX, indicating that the expression of NCLX is essential and sufficient to functionally complement mitochondrial Na^+/Ca^{2+} exchange. Despite the profound effect of NCLX expression on mitochondrial Ca^{2+} efflux, it did not affect the steady-state resting mitochondrial Ca^{2+} level. The relatively low affinity of the mitochondrial exchanger to Ca^{2+} (10) or the effective phosphate-based mitochondrial Ca^{2+} buffering system that is capable of keeping steady-state Ca^{2+} concentration constant over a wide range by a reversible formation or dissociation of insoluble Ca^{2+}-phosphate precipitates may account for this effect (22). Hence, NCLX may be playing a similar role to plasma membrane members of the NCX family that primarily mediate and respond to rapid and robust Ca^{2+} changes rather than fine tuning of the steady-state Ca^{2+} level (23). (*iii*) Mitochondrial Ca^{2+} efflux mediated by NCLX is strictly Na^+-dependent and coupled to accelerated mitochondrial Na^+ influx. The strong endogenous Na^+ dependence of Ca^{2+} efflux that we have assayed in permeabilized cells (Fig. 5) indicates that NCLX is the dominant Ca^{2+} extruding transporter in these cells. Further studies comparing the activity of NCLX and the H^+/Ca^{2+} exchanger are required to address their specific roles in distinct cell types and tissues. (*iv*) Replacement of a single residue in the putative catalytic α-domain of NCLX was sufficient to fully block mitochondrial Ca^{2+} efflux. The role of the α-domains as the catalytic core of NCX members is firmly established and hence this finding will provide the basis and rational for further studies on the unique and common structural/functional properties of the mitochondrial Na^+/Ca^{2+} exchanger. (*v*) NCLX is fully inhibited by the mitochondrial Na^+/Ca^{2+} exchange inhibitor CGP-37157 and (*vi*) NCLX mediates Li^+/Ca^{2+} exchange, a functional property that, among NCX proteins, is shared exclusively with the mitochondrial exchanger. Thus, the mitochondrial localization, the link between NCLX expression/inactivation with mitochondrial exchange activity, and its functional properties all indicate that NCLX is the mitochondrial exchanger.

Our results show that endogenous expression of NCLX in HEK 293 cells, as well as in other cells studied, is most prominent in mitochondria (Fig. 1); this is also seen in cells overexpressing the murine NCLX isoform. Thus, the enhanced plasma membrane Ca^{2+} transport activity that was previously found in HEK-293 cells ectopically expressing the human isoform of NCLX (7) is probably linked to a "spillover" of NCLX to the plasma membrane, a phenomenon commonly linked to strong overexpression in HEK-293 and other cell types (24, 25). Importantly, this spillover of the human NCLX protein to the plasma membrane fortuitously provided us with the seminal finding that NCLX mediates Li^+/Ca^{2+} exchange

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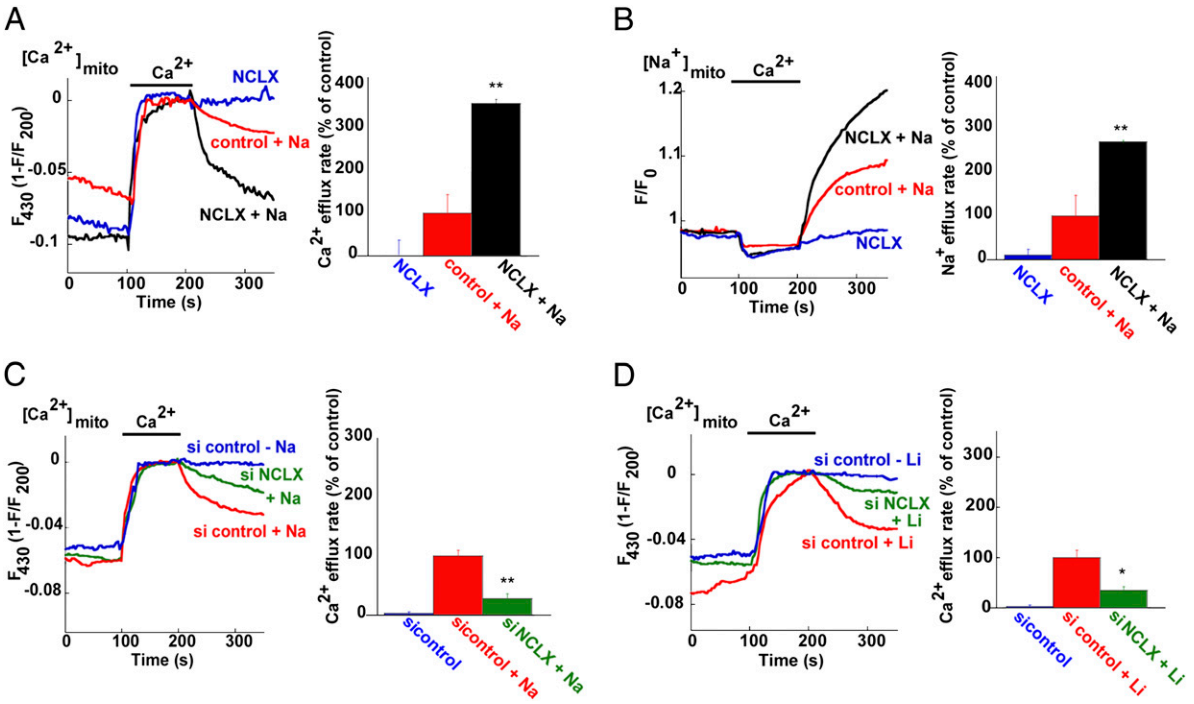

Fig. 5. The mitochondrial Na^+- or Li^+-dependent Ca^{2+} exchange is mediated by NCLX. (*A*) Traces and rates of Na^+-dependent mitochondrial Ca^{2+} efflux. Mitochondrial Ca^{2+} levels were recorded by monitoring RP-mt fluorescence in HEK-293 cells overexpressing either the human NCLX isoform (*NCLX*) or vector alone (*control*) and coexpressing RP-mt. Experiments were conducted on digitonin-permeabilized cells (see *Materials and methods*). Mitochondrial Ca^{2+} uptake was induced by superfusion with Na^+-free solution (replaced by $NMDG^+$) containing Ca^{2+} (60 μM). Ca^{2+} efflux was monitored following superfusion in Ca^{2+}-free solution in the presence or absence of Na^+. Note that the Ca^{2+} efflux was strictly Na^+-dependent and was enhanced by the expression of NCLX ($n = 8$, $^{**}P < 0.01$). (*B*) Traces and rates of Na^+-dependent mitochondrial Na^+ influx. Mitochondrial Na^+ levels were measured in cells loaded with the Na^+-sensitive dye CoroNa Red (see *Materials and methods*) as in *A*. Note the enhanced and reciprocal nature of the Na^+ and Ca^{2+} transport rates, mediated by mitochondrial NCLX ($n = 8$, $^{**}P < 0.01$). (*C*) Traces and rates of Na^+-dependent mitochondrial Ca^{2+} efflux in cells transfected with either siNCLX or a scrambled siRNA (*si control*). The same experimental paradigm described in *A* was applied. Silencing of NCLX eliminated mitochondrial Na^+-dependent Ca^{2+} efflux ($n = 11$, $^{**}P < 0.01$). (*D*) Traces and rates of Li^+-dependent mitochondrial Ca^{2+} efflux in cells transfected with either siNCLX or si control, using Li^+-containing solution (replacing Na^+; $n = 11$; $^{*}P < 0.05$). Silencing of NCLX also diminished Li^+-dependent Ca^{2+} efflux.

and prompted us to investigate if NCLX is the mitochondrial exchanger. We do not rule out, however, the possibility that NCLX or its spliced isoforms may also be found in other cellular compartments (7, 9). Indeed, NCX members are often found at multiple cellular sites. Among them, NCX1 is found both in the plasma membrane and nucleus (26). Dual localization is also shared by other mitochondrial proteins such as several members of the cytochrome P450 family that are targeted to both the ER and mitochondrial compartments (27, 28) and the potassium channel, KCa3.1, that can be found in the mitochondria or plasma membrane (29).

Our finding that NCLX is the mitochondrial Na^+/Ca^{2+} exchanger is of particular interest in light of the intense expression of NCLX in skeletal muscle, stomach, and pancreas (7) and the critical role played by the mitochondrial Ca^{2+} transport in these organs. A remarkable feature of mitochondrial Ca^{2+} transport in skeletal muscle is the ultrafast Ca^{2+} efflux rate (30), which equals the rate of influx mediated by the uniporter; compared with the 2 to 3 orders of magnitude slower efflux rate found in cardiomyocytes. The dramatically higher expression of NCLX in skeletal muscle versus cardiac tissue further links the expression of NCLX to mitochondrial Ca^{2+} efflux and suggests that expression of NCLX is a major rate limiting determinant of the uniquely fast mitochondrial Ca^{2+} transport in skeletal muscle. Smooth muscle accounts for the largest mass of tissue in the stomach, and it is therefore conceivable that the intense NCLX expression in the stomach results from its ubiquitous expression in smooth muscle (7). The essential role of the mitochondrial Na^+/Ca^{2+} exchanger in refilling smooth muscle Ca^{2+} stores and in facilitating the Ca^{2+} sparks leading to muscle contraction is documented (31, 32) and agrees well with the high expression of NCLX in this tissue. A role of mitochondrial Na^+/Ca^{2+} exchange in insulin secretion is of profound physiological interest particularly because of the link between mitochondrial Ca^{2+} levels and the rate of ATP synthesis, but is controversial because of potential interference of CGP-37157 with the L-type Ca^{2+} channel activity (16). In the exocrine pancreas, mitochondria have a well documented role in limiting the apical to the basolateral propagation of Ca^{2+} waves and in controlling plasma membrane Ca^{2+} influx (33). Much has to be learned on the specific role of the mitochondrial exchanger in the function of endocrine and exocrine pancreas, and the present study will provide the molecular tools required for this goal. Finally, the profound change in mitochondrial Ca^{2+} exchange activity observed for example in a neuronal Parkinson model (34) underscores the pathophysiological relevance of NCLX regulation. Hence, the identification of the mitochondrial Na^+/Ca^{2+} exchanger will provide insight on the molecular basis underlying these processes.

Materials and Methods

Fluorescent Measurements of Intracellular Ions. Cell culture and transfection procedures are described in SI Methods. Cytosolic Ca^{2+} levels were recorded from Fura-2–loaded cells, excited at wavelengths of 340 and 380 nm, and imaged with a 510 nm long-pass filter as previously described (7). Mitochondrial Ca^{2+} or H^+ levels were monitored in cells transiently expressing the RP-mt protein at excitation wavelengths of 430 nm [i.e., Ca^{2+}-sensitive wavelength (12), presented as F_{430} $(1\text{-}F/F_0)$] and 485 nm [i.e., pH-sensitive wavelength (35, 36), presented as F_{485} F/F_0], respectively, and the emission collected using a 535-nm band-pass filter. In experiments in which digitonin-permeabilized HEK-293 cells were employed, F_{430} was normalized to the value obtained following the Ca^{2+}-loading phase ($F_{T\text{-}200}$). Mitochondrial Na^+

levels were monitored in CoroNa-Red (Molecular Probes)–loaded cells excited at 545 nm and imaged using a 630-nm long-pass filter. Unless stated otherwise, all experiments in nonpermeabilized cells were conducted using Ringer solutions containing 130 mM NaCl or NMDG (Na^+-free), 20 mM Hepes, 15 mM glucose, 5 mM KCl, and 0.8 mM $MgCl_2$, with the pH adjusted to 7.4. Ringer solution was supplemented with 0.5 or 2 mM $CaCl_2$ (for only experiments described in *SI Materials and Methods*; Figs. S2 and S4*F*) or in 0.5 mM EDTA and 40 μM ATP as indicated. Mitochondrial Ca^{2+} or Na^+ in digitonin-permeabilized (0.007% digitonin) cells were determined as previously described (37) in a buffer containing 220 mM sucrose, 10 mM Hepes, 5 mM succinate, 2.5 mM KH_2PO_4, 0.4 mM EGTA, and 1 μM cyclosporine A, with the pH adjusted to 7.4 with KOH. Following the addition of 60 μM Ca^{2+}, and Ca^{2+} loading of the mitochondria, 1 μM ruthenium red was added to eliminate a leak of Ca^{2+} via this pathway. Then 20 mM NaCl, NMDG Cl, or 30 mM LiCl were added as indicated.

For all single-cell imaging experiments, traces of averaged responses, recorded from 5 to 20 cells in each experiment, were analyzed and plotted using KaleidaGraph. The rate of ion transport was calculated from each graph (summarizing an individual experiment) by a linear fit of the change in the fluorescence (ΔF) over a period of 30 s following initiation of apparent efflux/influx (ΔF/dt). Rates from n experiments (as mentioned in legends to the figures) were averaged and displayed in bar graph format. Analysis with the *t* test was performed to determine significance when relevant.

Immunoelectron Microscopy. Brain sections were processed and imaged as described in *SI Materials and Methods*. Analysis of the distribution of NCLX in control and NCLX-overexpressing cells was performed blindly using the gold-labeled preparations for the EM. Each mitochondrion containing at least one gold particle was digitally-captured at ×25,000 magnification. Only one mitochondrion was imaged per cell section, with 20 cells overall (i.e., 20 mitochondria): 10 overexpressing and 10 controls. Only mitochondria that exhibited a clear outer membrane and cristae were selected for the analysis. Each cell was counted as an event, and we then compared the gold particle number for each event. Similar numbers of extramitochondrial gold particles (n = 1–2) were observed in approximately 40% of the sections obtained either from control or NCLX-overexpressing cells.

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