TRP-ML1 Regulates Lysosomal pH and Acidic Lysosomal Lipid Hydrolytic Activity*

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Abigail A. Soyombo‡, Sandra Tjon-Kon-Sang‡, Youssef Rbaibi‡, Enkelejda Bashllari‡, Jill Bisceglia‡, Shmuel Mualem‡, and Kirill Kiselyov‡

From the Department of Physiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390 and Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Mucolipidosis type IV (MLIV) is caused by mutations in the ion channel mucolipin 1 (TRP-ML1). MLIV is typified by accumulation of lipids and membranous materials in intracellular organelles, which was hypothesized to be caused by the altered membrane fusion and fission events. How mutations in TRP-ML1 lead to aberrant lipolysis is not known. Here we present evidence that MLIV is a metabolic disorder that is not associated with aberrant membrane fusion/fission events. Thus, measurement of lysosomal pH revealed that the lysosomes in TRP-ML1−/− cells obtained from the patients with MLIV are over-acidified. TRP-ML1 can function as a H+ channel, and the increased lysosomal acidification in TRP-ML1−/− cells is likely caused by the loss of TRP-ML1-mediated H+ leak. Measurement of lipase activity using several substrates revealed a marked reduction in lipid hydrolysis in TRP-ML1−/− cells, which was rescued by the expression of TRP-ML1. Cell fractionation indicated specific loss of acidic lipase activity in TRP-ML1−/− cells. Furthermore, dissipation of the acidic lysosomal pH of TRP-ML1−/− cells by nigericin or chloroquine reversed the lysosomal storage disease phenotype. These findings provide a new mechanism to account for the pathogenesis of MLIV.

MLIV is a lysosomal storage disease typified by the accumulation of lipids and membranous material in intracellular organelles, predominantly lysosomes (reviewed in Refs. 1 and 2). Earlier attempts to explain the accumulation of lipids in MLIV focused on hyperactive endocytosis (3). However, endocytosis was found to be normal in skin fibroblasts (SF) from patients with MLIV (4–6). In addition, preliminary experiments as part of the present work showed normal net uptake of the fluid phase marker 10 kDa Texas Red dextran by TRP-ML1−/− cells. MLIV results from mutations in the ion channel TRP-ML1. Several studies (7–9), including ours (10), have shown that TRP-ML1 resides in the lysosomes. TRP-ML1 is a non-selective cation channel with limited Ca2+ permeability (7, 11). The same group reported impaired Ca2+ signaling in SF from an MLIV patient with the ΔF405 mutation in TRP-ML1 (7). Based on these findings, it is suggested that TRP-ML1 is a lysosomal Ca2+ (Ca2+) release channel that regulates endosomal-lysosomal fusion. The impaired fusion limits access of lipids to lysosomal lipases with the ensuing accumulation of unprocessed lipids (7, 12).

However, the fusion model is not compatible with the finding that TRP-ML1 is a strong outward rectifying channel (10, 13). The outward rectification indicates that the primary direction of cation flow by this channel is into the lysosome rather than out of the lysosome. As shown below, TRP-ML1 does not affect cellular Ca2+ signaling or Ca2+ content in the lysosomes. An alternative mechanism for the cellular phenotype of MLIV was suggested by the effects of deletion of CUP-5, the Caenorhabditis elegans homologue of TRP-ML1 that resulted in the accumulation of lysosomal markers in late endosomes (9). Because mature lysosomes are formed from late endosomes (14–16), it has been proposed that TRP-ML1 regulates maturation of lysosomes from late endosomes (9, 17).

At least two observations are inconsistent with either model. Both models predict a dramatic change in the number of lysosomes in MLIV-affected cells. The fusion model predicts fewer lysosomal-endosomal fusion events and thus increased number of lysosomes. The recycling model implies dramatic reduction in the number of lysosomes. In the present work, we did not detect any difference in LysoTracker or LAMP1 staining between control and TRP-ML1−/− SF (see below). No apparent changes in lysosomal numbers can be seen in other published images (6, 7). Both models predict an accumulation of lipids in prelysosomal compartments. However, lipids accumulate in the lysosomes of MLIV (4–6) (see Fig. 9).

Previously we have identified TRP-ML1 as a processed lysosomal, monovalent-permeable ion channel (10). Here we show that TRP-ML1 controls lysosomal pH (pH7) and consequently lipid hydrolysis and metabolism, which may account for the cellular phenotype of MLIV.

**EXPERIMENTAL PROCEDURES**

**TRP-ML1−/− Cells**—SF from patients with MLIV (WG0909) and MLIII (WG0109) were from the Repository for Mutant Human Cell Strains and the Coriell Cell Repositories (GM02525, GM02527, GM02048). The genetic defects in WG0909, GM02525, and GM02527 patients have been described previously (18). SF (GM09404) from a patient with ceroid lipofuscinosis 2 (CLN2) and a patient with Tay-Sachs (TS) disease (GM111853) were from the Coriell Institute. SF from heterozygous relatives (WG0987 and WG0140) and WT fibroblasts were used as controls. SF were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, and non-essential amino acids. The TRP-ML1 expression constructs have been described previously (10).

**Cell Fractionation**—Control and TRP-ML1−/− cells were washed twice with PBS and once with homogenization buffer (20 mM tricine, pH 7.6, 0.25 M sucrose, and 1 mM EDTA) and scraped and Dounce-homogenized 20 times followed by passing five times through a
23-gauge needle. The homogenate was centrifuged for 10 min at 400 \times g, and 1 mg of each post-nuclear supernatant was layered over a discontinuous gradient consisting of a 1.2-ml cushion of 2.5 M sucrose and 8.5 ml of an 18% Percoll in homogenization buffer. The gradient was centrifuged for 60 min at 67,000 \times g_{max} in a Sorvall T1270 rotor. The sucrose cushion and ten 1-ml fractions were collected from the bottom of the tube and the Percoll was removed by centrifugation for 40 min at 70,000 rpm in a Beckman TL 100.3 rotor. The samples were adjusted to 0.5% Triton X-100, passed five times through a 25-gauge needle, and incubated on ice for 30 min. Fractions were assayed for palmitoyl-CoA hydrolysis and immunoblots for TRP-ML1.

**Assays of Lipase Activity**—Total lipase activity was quantitated using the substrates palmitoyl-CoA and p-nitrophenyl-palmitate. [3H]Palmitoyl-CoA hydrolysis was measured as the release of [3H]palmitate after an extraction with Dole’s reagent. p-Nitrophenyl-palmitate hydrolysis was measured as the release of p-nitrophenol, which was monitored by absorbance at 400 nm. The reaction mixture contained 100 \mu M substate, 100 mM Hepes, and cell lysate in a final volume of 100 \mu L. The pH was adjusted between 4 and 9 by using sodium acetate and Hepes or Tris/Cl buffers as required. [3H]Palmitoyl-CoA was synthesized using [3H]dipalmitoylphosphatidylcholine.

**Thin-layer Chromatography**—Cells were labeled for 24 h with [3H]palmitic acid, washed with PBS, and extracted into 6 volumes of chloroform/methanol (1:1, v/v). The organic phase was washed once with PBS, and the same amount of lipid (based on the same number of counts) was analyzed by high performance thin layer chromatography using the following solvents. Phospholipids were separated by developing the plates with chloroform/methanol/ammonia (60:40:9, v/v/v), general and neutral glycolipids by developing with chloroform/methanol/water (65:25:4, v/v/v), and long chain fatty acids by hexane/ethyl acetate/acetetic acid (60:40:1, v/v/v).

**Electrophysiology**—All procedures were the same as described in Ref. 10, except that the pH of the bath solution was between 4 and 8.

**Immunocytochemistry**—Cells grown on coverslips were loaded with fluorescent indicators and placed in a perfusion chamber for experiments with live cells. Images were acquired with a Bio-Rad 1024 confocal microscope. The images were analyzed offline using the ImageJ software. The procedure described in Ref. 10 was used for experiments with fixed cells.

**Analysis of Acidine Orange (AO) and Oregon Green Fluorescence**—Cells on coverslips were loaded with AO by incubation with 1 \mu M AO for 10 min at 37 °C in standard bath solution (10) and analyzed using confocal microscopy. Acquisition parameters (iris, gain, offset, and laser intensity) were the same in all the experiments and were adjusted to nullify background fluorescence. Fluorescence was read with ImageJ software using two protocols: (a) a region of interest was drawn around the cell, and the fluorescence intensity was averaged; (b) fluorescence of individual organelles was estimated by placing the cursor over the image of the organelle and reading the corresponding intensity values. Both protocols yielded similar results, but the first protocol had been primarily used because it was more efficient. Statistical analysis of the intensity was performed in 5–10 separate experiments with 5 images in each experiment and 3–5 cells in each image. Estimation of the numbers of fluorescent particles in AO-loaded control and TRP-ML1\(^{-/-}\) cells was performed using the automated particle counting function of ImageJ.

**Measurement of [Ca\(^{2+}\)]**—For standard fluorescent recording, cells grown on coverslips were loaded with Fura2 in standard bath solution by incubation with 5 \mu M Fura2/AM for 30 min at 37 °C. The coverslips were placed in the perfusion chamber, and Fura2 fluorescence was recorded and calibrated as described in Ref. 22.

**Electron Microscopy**—Cells grown on plastic dishes were fixed by a 30-min incubation with a solution containing 2.5% glutaraldehyde in 0.1 M sodium cacodylate, washes with 0.1 M sodium cacodylate, post-fixed with a solution containing 1% OsO\(_4\) washed with PBS, and stained en bloc for 30 min with 2% uranyl acetate. Following the dehydration by immersion in 30–100% ethanol, the samples were embedded in resin by immersion in 30–100% resin:propylene oxide mixtures. Fixed samples were mounted on grids and analyzed with a JEOL 100CX transmission electron microscope.

**RESULTS AND DISCUSSION**

**TRP-ML1 Does Not Regulate Celluar Ca\(^{2+}\) Signaling**—Based on the comparison of agonist-mediated Ca\(^{2+}\) signaling in control fibroblasts and fibroblasts obtained from a patient carrying the \(\Delta F408\) mutation, it was concluded that TRP-ML1 controlled Ca\(^{2+}\) content in the endoplasmic reticulum and lysosomes/endosomes, and it was essential for agonist-mediated Ca\(^{2+}\) signaling (7). This required significant permeability of TRP-ML1 to Ca\(^{2+}\). The Ca\(^{2+}\) permeability of TRP-ML1 was evaluated by measuring the whole-cell current in HEK293 cells expressing TRP-ML1. Previously we have shown that overexpressing TRP-ML1 in HEK293 cells result in significant fraction of TRP-ML1 in the plasma membrane (10) permitting analysis of its channel properties. Fig. 1A shows that when added to the external medium, corresponding to the lumen of the lysosome, both Ca\(^{2+}\) and Mg\(^{2+}\) decreased the inward TRP-ML1 current. The inhibition by Ca\(^{2+}\) and Mg\(^{2+}\) was largely eliminated by 10 mM BAPTA (Fig. 1B) suggesting that either the inhibition was mediated by divalent ions in a plasma membrane microdomain or BAPTA was more efficient than EGTA in removing the inhibition by divalent ions. To further test the role of TRP-ML1 in Ca\(^{2+}\) signaling, we measured [Ca\(^{2+}\)]\(_{i}\) in control and TRP-ML1\(^{-/-}\) SF. Fig. 1C shows that bradykinin (Bk) similarly increased [Ca\(^{2+}\)]\(_{i}\) in control, TRP-ML1\(^{-/-}\), and control cells expressing the F46SL mutant. In Fig. 1D, Ca\(^{2+}\) content in the stores was evaluated by its complete discharge with ionomycin. Ca\(^{2+}\) contents in the stores of control and TRP-ML1\(^{-/-}\) SF were indistinguishable.

Another suggestion is that TRP-ML1 is a CaL channel that regulates Ca\(_{i}\) content and release (7). This predicts a significant difference in Ca\(_{i}\) between the WT and TRP-ML1\(^{-/-}\) cells. Ca\(_{i}\) was evaluated by incubating cells with 100 \mu M glycyl-L-phenylalanine B- naphthylamide, which bursts the lysosomes and releases their contents to the cytosol (23, 24). Fig. 1E shows that there was no detectable difference in Ca\(_{i}\) between control and TRP-ML1\(^{-/-}\) cells. Furthermore, overexpression of TRP-ML1 in SF or HeLa cells had no effect on Ca\(_{i}\) (Fig. 1F).

The results in Fig. 1 indicate that TRP-ML1 does not affect cellular Ca\(^{2+}\) homeostasis and is at variance with previous conclusion derived from experiments with cells carrying the \(\Delta F408\) mutation (7). It is likely that the results obtained with cells carrying the \(\Delta F408\) mutation reflect clonal behavior rather than an effect of TRP-ML1 on Gq-mediated Ca\(^{2+}\) signaling.

**TRP-ML1 Affects Lysosomal pH\(_{i}\)**—pH\(_{i}\) controls most lysosomal hydrolytic activities (25, 26). Localization of TRP-ML1 in the lysosomes (7–10) and its function as a monovalent cation channel (10, 13) raised the possibility that TRP-ML1 may regulate pH\(_{i}\). pH\(_{i}\) is established by the V-type H\(^{+}\) pump and a Cl\^- channel and can be as low as 5. The steep cytosolic-to-pH\(_{i}\) gradient can be visualized using AO, a fluorescent
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weak base that accumulates in acidic spaces (27). Thus, the larger the lysosomal to cytosolic pH gradient, the more AO will accumulate in the lysosomes resulting in a brighter AO fluorescence in over-acidified lysosomes. An increase in lysosome numbers is expected to also increase the numbers of fluorescent particles. In preliminary experiments, we found no intrinsic autofluorescence in control or fibroblasts of patients at the microscope setting used to detect AO fluorescence.

The role of TRP-ML1 in regulating pH_L was evaluated by measuring AO accumulation in WT and TRP-ML1^{-/-} fibroblasts. Fig. 2A shows that TRP-ML1^{-/-} accumulated about 40% more AO than control SF. AO accumulation is because low pH_L was ascertained by its complete dissipation by 5 μM nigericin, the H^+/K^+ exchange ionophore (not shown). In addition, Fig. 2B shows that AO accumulation in TRP-ML1^{-/-} cells was inhibited by the selective H^+ pump inhibitor bafilomycin. Fig. 2C shows that the increased lysosomal acidity in TRP-ML1^{-/-} SF was reversed by the expression of TRP-ML1. Estimation of the numbers of AO-positive organelles using an automated particle counting function showed no statistically significant differences in lysosomal numbers in control and TRP-ML1^{-/-} cells obtained from 10–20 separate experiments. B, AO accumulation in untreated TRP-ML1^{-/-} SF (left image) and SF treated with 1 μM bafilomycin (Baf) (right image). AU, absorbance unit. C, in control and TRP-ML1^-/- SF transfected with TRP-ML1. Note that the transfected TRP-ML1 decreased lysosomal AO accumulation in TRP-ML1^-/- cells. The columns show the summary of 8–12 experiments of OGD fluorescence under the indicated conditions.

FIGURE 2. TRP-ML1 regulates pH_L. A, confocal images of control and TRP-ML1^-/- SF loaded with 1 μM AO for 10 min at 30°C. The columns show statistical analysis of AO fluorescence intensity in control and TRP-ML1^-/- cells obtained from 10–20 separate experiments. B, AO accumulation in untreated TRP-ML1^-/- SF left image) and SF treated with 1 μM bafilomycin (Baf) (right image). AU, absorbance unit. C, AO accumulation in TRP-ML1^-/- SF transfected with TRP-ML1. Note that the transfected TRP-ML1 decreased lysosomal AO accumulation in TRP-ML1^-/- cells. D, SF expressing TRP-ML1 or TRP-ML1^F465L (F465L) were loaded overnight with OGD and chased for 24 h to incorporate the pH-sensitive dye into lysosomes. Transfected cells (marked by *) were identified by co-transfection with dsRed. The columns show the summary of 8–12 experiments of OGD fluorescence under the indicated conditions.
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ML1\(^{−}\)− cells with TRP-ML1, but not with the F465L mutant, increased OGD fluorescence (indicative of reduced acidity). Thus, in TRP-ML1-expressing cells, OGD fluorescence was 136 ± 9.11% of that in non-transfected cells in the same recording field, whereas in F465L-expressing cells the OGD fluorescence was 97 ± 5.81% of control cells. A higher OGD fluorescence in TRP-ML1-transfected lysosomes was dissipated by nigericin (not shown). In additional controls, no increase in OGD fluorescence was detected in TRP-ML1-expressing cells after 7 or 12 h of loading with OGD (99 ± 8% and 97 ± 8% of non-transfected cells, respectively). The latter findings also suggest that TRP-ML1 does not regulate the pH of late endosomes.

**TRP-ML1 Is Permeable to H\(^{+}\)**—As an ion channel, TRP-ML1 can reduce lysosomal acidification if it conducts H\(^{+}\). The H\(^{+}\) conductance of TRP-ML1 was measured by substituting all metal ions in the bath solution with NMDG to eliminate monovalent cation current and measuring the inward current at pH\(_{\text{intr}}\) between 8 and 4. Increasing external H\(^{+}\) concentration by reducing the pH from 8 to 5 modestly increased the inward current (Fig. 3A) and resulted in a marked shift in the reversal potential (\(E_{r}\)) from −70 to −10 mV in TRP-ML1 but not in control cells (inset in Fig. 3A and legend for Fig. 3B). Reducing external pH to 4 further shifted the \(E_{r}\), but the shift deviated from linearity probably because of partial inhibition of the current at this pH. Additional control is shown in Fig. 3C, where reducing pH to 5 and 4 inhibited the current by TRPC3 without shifting the \(E_{r}\).

The marked shift in the \(E_{r}\) indicates that TRP-ML1 is permeable to H\(^{+}\). A linear fit of the change in the \(E_{r}\) between pH 8 and 5 yielded an average shift of 15 mV/pH unit. Similar Nernstian slope values were reported for the highly selective voltage-activated H\(^{+}\) channels (29, 30). The sub-Nernstian value of the slope can be explained by limited permeability of TRP-ML1 to other ions and/or by depletion of H\(^{+}\) from near-plasma membrane domains (31, 32). The outward component of TRP-ML1 current in Fig. 3A shows that TRP-ML1 is permeable to Cs\(^{+}\). At pH 4, the constant field equation yields a pH \(^{-2}\)/pCs\(^{+}\) ratio of ~10\(^{8}\). At pH 7, where H\(^{+}\) depletion is expected to be most noticeable, the equation yields pH \(^{-2}\)/pCs\(^{+}\) of ~10\(^{8}\). Similar range of permeability ratios was reported for the voltage-activated H\(^{+}\) channels (reviewed in Ref. 33).

The small amplitude of the H\(^{+}\) current is because of the outwardly rectifying nature of TRP-ML1 and the low absolute concentration of H\(^{+}\) at pH 5.

The TRP-ML1 permeability for H\(^{+}\) and its inward rectification suggest a possible role of TRP-ML1 as a pH\(_{e}\) “safety valve,” i.e. TRP-ML1 can slowly dissipate high H\(^{+}\) concentration without compromising lysosomal H\(^{+}\) homeostasis. This proposed role of TRP-ML1 is depicted in the model in Fig. 3D.

**Role of TRP-ML1 in Lipid Trafficking**—MLIV is characterized by abnormal accumulation of membranous lipid material in the cells of patients (4, 34—36). To study the role of TRP-ML1 in lipid trafficking, we followed the time course of BODIPY-55-lactosylceramide (LacCer) uptake during a 15- or 30-min load and 5–60-min chase in WT and TRP-ML1\(^{−}\)− SF. To visualize the lysosomes, the cells were also labeled with 1 \(\mu M\) Lysotracker Red. Endocytosis of the lipid measured after 15-min load appeared normal in all cell types. Fig. 4A shows that LacCer begins to accumulate in the Golgi of control cells after 30-min chase, whereas the majority of the lipids was in the lysosomes of TRP-ML1\(^{−}\)− cells even after a 60-min chase. Analysis of the overlap between LacCer and Lysotracker shows that after a 60-min chase about 50% of LacCer was in the lysosomes of TRP-ML1\(^{−}\)− cells (Fig. 4C). Stimulation of the cells with BK did not accelerate LacCer traffic (Fig. 4B and C) further questioning a deficient Ca\(_{\text{L}}\) release as the primary cause of MLIV.
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Further evidence for lipid accumulation in the lysosomes of TRP-ML1−/− cells was obtained by staining the cells with the antibiotic filipin, which stains unesterified cholesterol. Fig. 4D shows intense and widespread filipin staining of TRP-ML1−/− cells, indicating accumulation of cholesterol in the lysosomes of these cells.

**TRP-ML1 Regulates Lipid Hydrolysis and Metabolism**—To address directly the role of TRP-ML1 in lipid hydrolysis, total soluble lipases were extracted, and lipid hydrolysis was analyzed in two WT and four independent TRP-ML1−/− SF lines obtained from unrelated patients with MLIV. SF from patients with late infantile neuronal CLN2 and TS diseases were used as additional controls. Fig. 5A shows normal expression of TRP-ML1 in WT, CLN, and TS cells and absence of the channel in all TRP-ML1−/− clones. Vigorous disruption of the cells released better than 90% of the lipid hydrolytic activity to the soluble fraction. The remaining 10% is associated with the particulate fraction and was unaltered in all cells (not shown). Because TRP-ML1 is found only in the particulate fraction, this indicates that TRP-ML1 has no or negligible lipase activity. Remarkably, the soluble palmitoyl-CoA hydrolysis of all four TRP-ML1−/− lines was only 40% of that measured in WT cells. This was not because of impaired lysosomal storage since hydrolysis was

**Lipid Trafficking in Control and TRP-ML1−/−**—SF, A lipid trafficking was tested by following LacCer trafficking in control and TRP-ML1−/− SF using confocal microscopy. Cell were loaded with 5 μg/ml BODIPY-CS LacCer complexed to bovine serum albumin for 15 min and chased for 15–60 min at 19°C. Note perinuclear localization of the lipid in control cells and lysosomal localization in TRP-ML1−/− cells. The images are representative of 3–4 experiments (5–10 individual cells analyzed in each experiment) similar to those in A and B. AU, absorbance unit. C, shows staining of WT and TRP-ML1−/− cells with filipin. As summarized by the columns, TRP-ML1−/− cells accumulated about three times more filipin than WT cells.

**Cleavage of TRP-ML1 is Required to Activate Palmitoyl-CoA Hydrolysis**—A control SF were treated with 10 μM E64d for 24, 48, or 72 h, and extracts from untreated (Ctrl) and treated (E64d) cells were used to analyze expression of native TRP-ML1 using αN1 (upper blot) to measure palmitoyl-CoA hydrolysis. The results are the mean ± S.E. of triplicate determination and are representative of three experiments with similar results. *, difference from WT of the same incubation period and 24 h treatment at p < 0.05. **, difference from WT at 48 h treatment at p < 0.01. ***, difference from WT at 72 h treatment at p < 0.001.

![FIGURE 4](image1.jpg)

**FIGURE 4. Lipid Trafficking in Control and TRP-ML1−/−**

![FIGURE 5](image2.jpg)

**FIGURE 5. The Role of TRP-ML1 in Lipid Hydrolysis.** A, the blot shows expression of TRP-ML1 in the indicated cell lines. ML1−/− is clone WG0909, ML1−/−2 is clone GM02525, ML1−/−3 is clone GM02527, and ML1−/−4 is clone GM02048. CLN is a clone from a patient with neuronal CLN, and TS is a clone from a patient with TS disease. The columns show the palmitoyl-CoA hydrolase in the extract of lysosomal lipases prepared from these cells. B, restoration of palmitoyl-CoA hydrolysis to TRP-ML1−/− cells was measured in extracts prepared from WT and TRP-ML1−/− transfected with vector or TRP-ML1. The results are the mean ± S.E. of three experiments. C, hydrolysis of p-nitrophenyl-palmitate (pNPP), cholesterol-oleate (ChoOl), triolein, and dipalmitoylphosphatidylcholine (dPPC) was measured in the solubles (pNPP, ChoOl, triolein) or particulate (dPPC) fractions prepared from WT and TRP-ML1−/− cells, and the activity in TRP-ML1−/− extracts was calculated as % of the respective controls.

![FIGURE 6](image3.jpg)

**FIGURE 6. Cleavage of TRP-ML1 is Required to Activate Palmitoyl-CoA Hydrolysis.** A, control SF were treated with 10 μM E64d for 24, 48, or 72 h, and extracts from untreated (Ctrl) and treated (E64d) cells were used to analyze expression of native TRP-ML1 using αN1 (upper blot) to measure palmitoyl-CoA hydrolysis. The results are the mean ± S.E. of triplicate determination and are representative of three experiments with similar results. *, difference from WT of the same incubation period and 24 h treatment at p < 0.05. **, difference from WT at 48 h treatment at p < 0.01. ***, difference from WT at 72 h treatment at p < 0.001.
normal or only modestly reduced in CLN and TS extracts. This indicates chronic inactivation of lipid hydrolysis in MLIV cells.

In Fig. 5B, the role of TRP-ML1 in lipid hydrolysis was further verified by expressing the channel in TRP-ML1<sup>−/−</sup> cells, which increased the palmitoyl-CoA hydrolysis by 31 ± 1%. Normalization for transfection efficiency of 23 ± 5% indicates that TRP-ML1 completely restores palmitoyl-CoA hydrolysis to TRP-ML1<sup>−/−</sup> cells.

To further analyze lipid hydrolysis, we used four additional substrates: p-nitrophenyl-palmitate for total lipase activity, triolein to assay general lipases, cholesteryl-oleate to assay cholesterol esterase, and dipalmitoylphosphatidylcholine to assay the particulate phospholipase A<sub>2</sub> activity. Similar reduction in hydrolytic activity was observed with all the substrates for the soluble lipases of TRP-ML1<sup>−/−</sup> cells (Fig. 5C). Dipalmitoylphosphatidylcholine activity was found only in the particulate fraction and was the same in WT and TRP-ML1<sup>−/−</sup> cells (Fig. 5C). These findings suggest a global reduction in the lysosomal lipase activity in TRP-ML1<sup>−/−</sup> cells. Hence, the acidic pH<sub>L</sub> in TRP-ML1<sup>−/−</sup> cells resulted in the inactivation of lipase activity and not merely shifting pH<sub>L</sub> away from the optimum for lysosomal lipases.

Because cleavage of TRP-ML1 inhibited channel function (10), we tested how the inhibition of cleavage affects the lipase activity. Significantly, Fig. 6A shows that progressive inhibition of cleavage by incubating WT fibroblasts for 24, 48, and 72 h with E64d results in an increased full-length TRP-ML1 and a corresponding inhibition of lipase activity. As a control, Fig. 6B shows that treatment of TRP-ML1<sup>−/−</sup> fibroblasts with E64d for 48 h has no effect on lipase activity. Expression of TRP-ML1 in TRP-ML1<sup>−/−</sup> cells restored palmitoyl-CoA hydrolysis, and inhibition of the cleavage of the expressed TRP-ML1 inhibited restoration of lipase activity (Fig. 6B).

To determine whether the lysosomal lipases were predominantly affected in MLIV, microsomes were prepared from WT and TRP-ML1<sup>−/−</sup> cells and were fractionated on an 18% Percoll gradient. Organelles expressing TRP-ML1 (lysosomes) were recovered mostly in the heaviest fraction 1 (Fig. 7A), whereas palmitoyl-CoA hydrolysis was found in fractions 1 and 2 of WT cells but only in fraction 2 of TRP-ML1<sup>−/−</sup> cells (Fig. 7B). The pH profile of the lipase activity revealed that fraction 1 contained most of the lipases active at acidic pH (Fig. 7C), whereas fraction 2 contained lipases with pH optimum between 6 and 7 (Fig. 7D). Notably, only the acidic lipases in the lysosomal fraction were inactive in TRP-ML1<sup>−/−</sup> cells.

Next we asked whether the reduced lipase activity is accompanied by aberrant lipid processing. The lipids of WT and TRP-ML1<sup>−/−</sup> cells were labeled by an overnight incubation with <sup>3</sup>H]palmitate to a pre-steady state level so that the difference in lipid synthesis could be resolved better. Chromatography with solvents that separated phospholipids, general and neutral glycolipids, and long chain fatty acids revealed multiple differences in lipids synthesized during the labeling period (Fig. 8). Dissipation of Acidic pH<sub>L</sub> in TRP-ML1<sup>−/−</sup> Cells Reverses the MLIV Phenotype—An important implication of the observation that chronic acidic pH<sub>L</sub> is a primary cause of MLIV may be the possibility of treating...
the disease with compounds that decrease the lysosomal acidity. The feasibility of such an approach is demonstrated in Fig. 9. Because long incubation with bafilomycin proved to be toxic to the cells (Fig. 9A), SF from normal and MLIV patients were incubated with the H\(^+\)/K\(^+\) ionophore nigericin (0.2 \(\mu\)M) for 4 days, and their morphology was analyzed by electron microscopy. Untreated SF from patients with MLIV contained large numbers of dark inclusions filled with enfolded membranes. The number of the inclusions dramatically decreased in cells treated with nigericin. The specificity of the effect of dissipating pHi to the MLIV phenotype was tested by measuring the effect of nigericin on SF obtained from a patient with MLIII (pseudo-Hurler polydystrophy). Although the morphological phenotype of MLIV and MLIII are very similar, nigericin did not reverse the MLIII phenotype.

Chloroquine is a weak base that accumulates in acid spaces and dissipates pHi. High concentrations of chloroquine are toxic resulting in cell death. Indeed, cells from patients with MLIV are highly sensitive to chloroquine (37), consistent with more acidic pHi in these cells. However, with the aim of testing the potential use of chloroquine for treatment of MLIV, we tested whether chloroquine can also reverse the MLIV phenotype. Testing several concentrations revealed that it was safe and sufficient to treat the cells with as little as 10 nM chloroquine for 4 days, and such a treatment partially reversed the MLIV phenotype (Fig. 9B).

To provide a statistical estimate of the number of MLIV-specific membrane inclusions before and after the chloroquine treatment, images representing approximately half of a cell were thresholded using ImageJ software to render clearly distinguishable binary pictures of the inclusions (Fig. 9C). This was followed by counting the particles using the particle counting function of ImageJ. To avoid a background bias, the minimal size of the particle was set at 100 pixels (~0.1 \(\mu\)m) in diameter. The column in Fig. 9C shows that treatment with chloroquine reduced the inclusions by about 65%. Although the cells were treated with only 10 nM chloroquine, Fig. 9D shows that this treatment was sufficient to reduce AO accumulation, indicating partial dissipation of the acidic pHi by chloroquine.

The findings in Fig. 9 reinforce the conclusion that MLIV is caused by chronically acidic pHi and offer a potential strategy for the development of modalities to treat MLIV based on inhibitors of lysosomal acidifiers. Chloroquine and derivatives and the novel V-type H\(^+\) pump inhibitors described recently might be considered for such a treatment (38).

Conclusions—The present findings together with characterization of TRP-ML1 (10) provide a novel mechanism to explain the MLIV pathogenesis and suggest a new approach for developing treatment for the disease. The phenotype of MLIV led to the prevailing mechanism for the role of TRP-ML1 in MLIV, in which TRP-ML1 controls membrane utilization by regulating membrane fusion/fission events (7, 9, 17). Our findings do not support such mechanisms but show marked inactivation of lipases in TRP-ML1 \(^{-/-}\) cells, indicating that MLIV is a metabolic rather than a trafficking disease.

At least two mechanisms can account for the inactivation of lipases by over-acidification of the lysosomes. Chronic over-acidification may denature and inactivate the lysosomal lipases. Alternatively, because assembly of lysosomal proteins into functional complexes has been shown to depend on lysosomal acidity (39, 40), over-acidification may disrupt formation of the complexes.

The present work suggests a novel role for TRP-ML1 in regulating lysosomal function. When intact in the lysosomes, TRP-ML1 provides a H\(^+\) leak pathway to prevent over-acidification of the lumen. When further acidification is needed to activate lipases, proteases or other lysosomal functions, TRP-ML1 is cleaved by a CatB-dependent pathway that may be activated by very acidic pHi. Continued insertion and cleavage of TRP-ML1 results in cycles of decrease and increase in pHi that is needed for the normal functioning of lysosomal lipases. MLIV-associated mutations in TRP-ML1 disrupt regulation of pHi and lead to aberrant activity of lysosomal lipases and the MLIV phenotype.

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