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A role for the Ca²⁺ channel TRPML1 in gastric acid secretion, based on analysis of knockout mice

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Abstract

Background & Aims—Mutations in TRPML1, a lysosomal Ca²⁺-permeable TRP channel, lead to mucopolidosis type IV (MLIV), a neurodegenerative lysosomal storage disease. An unusual feature of MLIV is constitutive achlorhydria. We produced *Trpml1*^{−/−} (null) mice to investigate the requirement for this protein in gastric acid secretion.

Methods—*Trpml1*-null mice were generated by gene targeting. The expression of *Trpml1* and its role in acid secretion by gastric parietal cells were analyzed using biochemical, histological, and ultrastructural approaches.

Results—*Trpml1* is expressed by parietal cells and localizes predominantly to the lysosomes; it was dynamically palmitoylated and dephosphorylated *in vivo* following histamine stimulation of acid secretion. *Trpml1*-null mice had significant impairments in basal and histamine-stimulated gastric acid secretion and markedly reduced levels of the gastric proton pump. Histological and ultrastructural analyses revealed that *Trpml1*^{−/−} parietal cells were enlarged, had multivesicular and multi-lamellated lysosomes, and maintained an abnormal intracellular canaliculi membrane. The intralysosomal Ca²⁺ content and receptor-mediated Ca²⁺ signaling were, however, unaffected in *Trpml1*^{−/−} gastric glands, indicating that *Trpml1* does not function in the regulation of lysosomal Ca²⁺.

Conclusions—Loss of *Trpml1* causes reduced levels and mislocalization of the gastric proton pump and alters the secretory canaliculi, causing hypochlorhydria and hypergastrinemia. The lysosomal enlargement and defective intracellular canaliculi formation observed in *Trpml1*^{−/−} parietal cells indicate that *Trpml1* functions in the formation and trafficking of the tubulovesicles.

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This study provides direct evidence for the regulation of gastric acid secretion by a TRP channel; TRPML1 is an important protein in parietal cell apical-membrane trafficking.

Keywords

Apical membrane trafficking; Mucolipin-1; Vesicles; Stomach

Introduction

Gastric acid secretion originates in parietal cells, which are highly specialized epithelial cells located in the stomach mucosa, and it is driven by the gastric proton pump, H^+K^+ -ATPase.¹ The intracellular morphology of the parietal cell is unique, with numerous mitochondria and a vast array of vesicular and/or short tubular structures, the tubulovesicles (TVs), which sequester H^+K^+ -ATPase in an intracellular compartment within the resting cell. Upon secretagogue stimulation and cAMP-dependent signaling, parietal cells undergo striking morphological transformations involving the translocation and fusion of H^+K^+ -ATPase-rich TVs with the apically-directed intracellular canaliculi, resulting in an elaborate system of deeply invaginated apical secretory surface.^{2–3}

Following secretagogue removal, parietal cells transition to the resting conformation through retrieval and reinternalization of H^+K^+ -ATPase-rich membranes from the expanded apical membranes followed by reformation of TVs through endocytosis.^{4–5} The molecular machinery governing the trafficking of H^+K^+ -ATPase include several regulatory proteins such as SNAP-25, VAMP-2, syntaxin 1 and 3, the rab GTPases namely rab11a and rab25,^{6–7} clathrin and its adaptor proteins^{6, 8} and ezrin.⁹ Thus, proteins that control the sorting of H^+K^+ -ATPase and vesicle fusion events have been purified from TVs. However, the identity of ion channels that regulate ion fluxes during these parietal cell apical membrane fusion and fission events remain unknown.

A unique clinical abnormality seen in mucopolipidosis type IV (MLIV, OMIM: #252650) is achlorhydria with consequent hypergastrinemia.^{10–11} MLIV is an autosomal recessive, neurodegenerative lysosomal storage disorder that is characterized by severe mental and psychomotor retardation.¹² Mutations in the gene encoding TRPML1, a lysosomal cation channel, cause MLIV.^{13–14} TRPML1, together with TRPML2 and TRPML3 form the TRPML subfamily and are localized primarily within the endo-lysosomal pathway.¹⁵

Morphological and biochemical studies of MLIV cells have revealed an accumulation of lamellated membranous storage bodies^{16–17} and delayed exit of endocytosed lipids from lysosomes.¹⁸ MLIV fibroblasts show impaired lysosomal lipid hydrolase activity,^{19–20} lysosomal exocytosis²¹ and autophagosome-lysosome fusion.²² Furthermore, CUP-5, the *C.elegans* orthologue of TRPML1, is implicated in lysosomal reformation from late endosome-lysosome hybrid organelles^{23–24} suggesting a role for TRPML1 in lysosomal trafficking. The biophysical properties of TRPML1 remain to be clarified. TRPML1 has been described as a pH-regulated monovalent cation channel,²⁵ a proton-leak channel that regulates lysosomal pH,¹⁹ and a lysosomal Fe^{2+} release channel.²⁶ Gain-of-function mutations in TRPML1 were associated with a monovalent cation- and Ca^{2+} -permeable channel,²⁷ suggesting that TRPML1 may function physiologically as a Ca^{2+} channel.

We investigated the role of Trpml1 in gastric acid secretion using a new mouse model of MLIV. Our studies show that Trpml1 is dephosphorylated and palmitoylated following histamine stimulation of acid secretion. Trpml1^{-/-} mice displayed markedly reduced levels of the gastric proton pump explaining the achlorhydria previously reported in MLIV patients. Notably, Trpml1^{-/-} parietal cells displayed an extensive network of enlarged

lysosomes and formed an abnormal H⁺K⁺-ATPase-containing intracellular canalicular membrane, indicating a lysosomal maturation blockade and an impaired apical membrane dynamics. These findings provide important insights into the role of TRPML1 in gastric acid secretion.

Materials and Methods

Antibodies

Monoclonal antibodies to gastric H⁺/K⁺-ATPase α - (1H9) and β -subunits (2B6) were from MBL. An affinity-purified antibody to mouse *Trpml1* was raised in rabbits with a peptide corresponding to amino acids 32–48 (N-terminus antibody) of *Trpml1* (GeneID: 94178). Antibodies to Snap-25, *Trpml1* (C-terminus antibody), phosphoserine-agarose (clone PSR-45) and β -actin were purchased from Sigma. Antibody to ezrin was from Cell Signaling. The anti-lamp-1 antibody (1D4B) developed by J. Thomas August was obtained from Developmental Studies Hybridoma Bank.

Generation of *Trpml1*-Deficient Mice

A *Trpml1* targeting vector was constructed using the plasmid, pKO Scrambler NTKV-1901. A 2.5 kb short fragment corresponding to the promoter region of *Trpml1* and its upstream sequences and a 5.5 kb fragment that include sequences corresponding to exons 3 to 12 were amplified by PCR using primers that were derived from Genbank Accession # NC_000074.5. The tetracycline-responsive transcriptional activator (tTA) was placed downstream of the promoter region of *Trpml1*. Murine embryonic stem (ES) cells were electroporated with the linearized targeting vector and screened for homologous recombination by Southern blotting. Two correctly targeted ES clones were selected, expanded and injected into C57BL/6 mouse blastocysts to generate chimeric mice that were bred to obtain germline transmission. The experiments described in this study were performed using *Trpml1* wild-type and knockout littermates on a C57BL/6 X 129SV mixed background. Animal procedures were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Southern Blot Analysis and Genotyping

Genomic DNA from ES cells was digested with Hind III, separated by agarose gel electrophoresis and blotted onto Nytran membrane which was hybridized with a radiolabeled 800-bp 5' probe (Fig. 1A). Genotype analysis of tail DNA was performed by PCR.

Northern Blot Analysis

Total RNA was isolated from WT, heterozygotes and *Trpml1*^{-/-} mice brain and glandular stomach and electrophoresed on a formaldehyde-agarose gel, transferred to Nytran membrane and hybridized with radiolabeled DNA fragments.

Gastric Acid and Serum Gastrin Analysis

Sex-matched littermates were fasted overnight but allowed free access to water. Following subcutaneous injection of histamine for 40 min (2 μ g/g body weight), mice were killed, their stomach contents washed into 2 ml of saline and centrifuged. The pH of the supernatant was measured with a pH electrode and acid content, determined as the volume of 10 mM NaOH required to titrate the supernatant to the starting saline pH, was expressed as μ Eq/g stomach wet weight. Gastrin in serum from fasted WT and *Trpml1*^{-/-} littermates was analyzed using a radioimmunoassay kit from MP Biochemicals.

Light and Transmission Electron Microscopy

Mice were anesthetized using Avertin and perfused transcardially with PBS followed by a solution of 4% paraformaldehyde in PBS for light and confocal microscopy or a solution of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for electron microscopy. Tissues from the glandular stomach were post-fixed in 1% buffered osmium tetroxide, stained en bloc in 2% uranyl acetate, dehydrated with ethanol, and embedded in EMbed-812 resin. Stomachs from postnatal day 5 mice were immersion-fixed in 2.5% glutaraldehyde. Thick sections (500 nm) were stained with 1% toluidine blue. Thin sections (80 nm) were stained with 2% uranyl acetate and lead citrate. Images were acquired on a FEI Tecnai G² Spirit at 120 kV. To quantify cell size increases in *Trpml1*-deficient parietal cells, the distance from the apical membrane to the center of the basolateral membrane was measured in H&E stained paraffin sections and converted into μm using a calibrated slide.

Immunohistochemistry (IHC) and Confocal Immunofluorescence Microscopy

For IHC, paraffin sections from WT and *Trpml1*^{-/-} stomachs were deparaffinized, rehydrated and treated for 10 min in PBS containing 0.1% Triton, washed in PBS and incubated in 0.3% H₂O₂ in PBS for 30 min. After several rinses in PBS, the sections were incubated in 5% normal horse serum in PBS for 30 min at RT. Sections were then incubated with anti-lamp-1 antibody for 1 h at RT, washed and incubated with HRP conjugated secondary antibody for 30 min at RT. Sections were developed with a 0.05% 3,3'-diaminobenzidine/H₂O₂ solution and counterstained with hematoxylin. For immunofluorescence (IF), frozen sections were air-dried and treated with 0.1% Triton as described above. Following primary antibody incubations and washing in PBS, sections were incubated with either Alexa 488- or Alexa 594- conjugated secondary antibodies, washed and mounted in Prolong anti-fade (Invitrogen). F-actin staining was performed on frozen sections with Alexa 488-Phalloidin. Sections were examined by confocal laser-scanning microscope (Leica TCS SP5). Laser intensity and detector settings were kept constant for paired WT and *Trpml1*^{-/-} sequential image acquisitions.

Acyl-biotinyl Exchange (ABE)

ABE²⁸⁻²⁹ was performed as previously described to evaluate *Trpml1* palmitoylation *in vivo*. Fasted mice were injected with or without histamine for the indicated times and the glandular stomachs from three pairs of WT or *Trpml1*^{-/-} littermates were homogenized in 10 ml buffer HB containing (in mmol/L) 50 Tris (pH 7.4), 150 NaCl, 1 EDTA, 1 PMSF, 1 NaF, 1 Na₃VO₄, 10 N-ethylmaleimide (NEM) and protease inhibitors (Roche). Crude microsomes were solubilized with lysis buffer (HB plus 1% Triton X-100, 0.5% sodium deoxycholate). Detergent extracts containing equal amounts of protein from WT or *Trpml1*^{-/-} microsomes were sequentially extracted with chloroform-methanol (CM), as previously described²⁹. Precipitated proteins were resuspended in buffer SB containing (in mmol/L) 50 Tris (pH 7.4), 5 EDTA, 10 NEM and 4% SDS and incubated overnight at 4°C. After CM extractions to remove NEM, precipitated proteins were then resuspended in 2 ml buffer SB (without NEM) and divided into two tubes. One tube was treated with hydroxylamine (HA) and biotin-HPDP by adding buffer containing (in mmol/L) 1000 HA (pH 7.4), 150 NaCl, 1 biotin-HPDP and 0.2% Triton X-100. The control tube was treated with 50 mmol/L Tris. Further CM extractions, capture of biotinylated proteins with streptavidin agarose and 10% TCA precipitation of eluted proteins were as previously described²⁹. The final pellet was subjected to protein assay to ensure equal protein loading, separated by SDS-PAGE and analyzed by immunoblotting.

Measurement of Intracellular and Intralysosomal Ca²⁺ in Gastric Glands

The mucosa was scraped from the glandular stomachs of 6–8 weeks old WT and *Trpm1*^{−/−} mice, finely minced and incubated with 0.25 mg/ml collagenase type 1A (Sigma) in extracellular medium (ECM) for 10 min at 37°C with shaking. The liberated glands were collected, washed in ECM and passed through a tissue strainer to obtain individual glands. Glands were loaded with Fura-2 by incubation with Fura-2AM for 30 min at RT and ratiometric imaging of intracellular Ca²⁺ was performed as previously described¹⁹. Glycyl-L-phenylalanine 2-naphthylamide (GPN) was used to release Ca²⁺ from lysosomes by inducing their selective swelling³⁰.

Immunoprecipitation, SDS-PAGE and Immunoblotting

Trpm1 immunoprecipitation was performed with anti-phosphoserine-agarose or anti-*Trpm1*. Immune complexes were washed with lysis buffer, analyzed by SDS-PAGE and blotted with antibody, as indicated. Membrane extracts from the glandular stomachs of WT and *Trpm1*-deficient littermates were analyzed by SDS-PAGE and immunoblotting.

Data Analysis

The results are expressed as means ± SEM. A Student's t-test was used to evaluate the statistical significance between two paired observations. *P* < 0.05 was considered statistically significant.

Results

Generation of *Trpm1*^{−/−} Mice

The mutant allele of *Trpm1* was derived by homologous recombination in ES cells by replacing exons 1, 2 and portions of exon 3 with tTA and the neomycin resistance gene (Fig. 1A). Correct placement of the targeting construct was confirmed by Southern blotting. Fig. 1B shows the expected wild-type fragment of 18 kb and an additional 15 kb which corresponds to the targeted *Trpm1* allele. Genotype analysis of DNA from litters of heterozygote matings (Fig. 1C) showed a 0.8 kb fragment and a 0.5 kb fragment corresponding to the wild-type and targeted alleles, respectively. Northern analysis of brain RNA (Fig. 1D) with probe corresponding to exons 12–14 of *Trpm1* confirmed the absence of *Trpm1* mRNA in KO mice. There was also an absence of the full length 65 kDa and the characteristically cleaved endogenous *Trpm1* protein³¹ in the stomach and brain (Fig. 1E). *Trpm1*^{−/−} mice exhibited normal growth and survival up to about 6 months of age at which time they showed growth retardation and hindlimb paralysis, and were sacrificed due to paralysis and weight loss. The mice described in this study reproduced the clinical and cellular features of MLIV.³²

Reduced Basal and Histamine-Stimulated Gastric Acid in *Trpm1*^{−/−} Mice

Constitutive achlorhydria has been described in MLIV.^{10–11} We determined baseline and histamine-stimulated stomach acid content in fasted WT and *Trpm1*^{−/−} littermates (n = 10). The basal acid content in 16 weeks old *Trpm1*^{−/−} mice was significantly lower than sex-matched WT littermates. The baseline gastric pH of *Trpm1*^{−/−} mice was 4.45 ± 0.3, which was 1.3 log units higher than 3.13 ± 0.1 in WT littermates (n = 10, *p* < 0.05, Fig. 2A). Following histamine injection, both genotypes increased acid output (Fig. 2B) with *Trpm1*^{−/−} mice decreasing their stomach pH to 3.4 ± 0.3 whereas wild-type stomach pH was 2.42 ± 0.1 (n = 12, *p* < 0.05), demonstrating that *Trpm1*^{−/−} mice are hypochlorhydric and not achlorhydric as reported in MLIV patients. Analysis of acid outputs at 8 and 24 weeks of age revealed that the loss of acid secretion in *Trpm1*^{−/−} mice was maximal by 8 weeks of age (Fig. 2C). In light of the hypochlorhydria in *Trpm1*^{−/−} mice, we looked at the

expression levels of the gastric proton pump subunits, H⁺K⁺-ATPase α - and β . Immunoblotting showed a marked reduction in the mature form of H⁺K⁺-ATPase α -subunit in Trpml1^{-/-} stomachs (Fig. 2D, asterisk, n = 6). In addition, a significant fraction of H⁺K⁺-ATPase α -subunit existed in an aggregated, high molecular weight forms that were retained in the gel wells and in the stacking gel (Fig. 2D, arrows), in Trpml1^{-/-} mice. The band below the asterisk represents a degradation product of H⁺K⁺-ATPase α .³³ The β -subunit also shows a reduction in protein levels in Trpml1^{-/-} stomachs (Fig. 2D, about 50%, n = 6) and the mRNA for H⁺K⁺-ATPase α -subunit was also reduced (Fig. 2E).

Trpml1 is Palmitoylated and Dephosphorylated Following Stimulation of Acid Secretion

To understand the role of Trpml1 in gastric acid secretion, first we looked at its expression in gastric glands by IF. Fig. 3A shows a typical punctate lysosomal staining of Trpml1 in parietal cells and high expression levels at the glandular luminal regions (arrows). Parietal cells were identified by their positive staining for H⁺K⁺-ATPase β -subunit (Fig. 3B). The basolateral membrane staining in Fig 3B is unrelated to H⁺K⁺-ATPase, since it was present when the H⁺K⁺-ATPase antibody was omitted. Chief cells (CC) adjacent to parietal cells showed low lysosomal expression of Trpml1, but high reactivity at the apical membranes. The distribution of Trpml1 and its colocalization with lamp-1, a lysosomal marker, is shown in Supplementary Fig. 1, indicating that Trpml1 localizes to the lysosomes in parietal cells as has been described in other cell types.³⁴ Next, we examined the palmitoylation status of Trpml1 in gastric glands by performing an acyl-biotinyl exchange (ABE) assay. The ABE method exchanges a biotin moiety for thioester-linked palmitate, thus allowing for the protein's capture with streptavidin agarose²⁹. TRPML1 has been previously reported to be palmitoylated at its C-terminal cytoplasmic tail in a heterologous expression system³⁵ and this modification increased its membrane attachment. Indeed, the cleaved C-terminal fragment of Trpml1 was palmitoylated in WT stomach extracts (Fig. 3D). The specificity of Trpml1 palmitoylation is shown by the absence of signal in Tris buffer treated control samples and in Trpml1^{-/-} extracts. Notably, stimulation of acid secretion augmented Trpml1 palmitoylation (Fig. 3E), demonstrating that Trpml1 palmitoylation, which may serve to anchor Trpml1 at the apical membranes, is dynamic and correlated with parietal cell activation. Occasionally, in over-exposed immunoblots, the 65 kDa full-length Trpml1 form was present in the palmitoylated fraction (data not shown), however, the most abundant Trpml1 species present in the palmitoylated fraction is the cleaved fragment. Snap-25, a palmitoylated SNARE protein that functions in tubulovesicle fusion,³⁶ verified equal protein loading. We also examined Trpml1 phosphorylation since protein kinase A-mediated Trpml1 phosphorylation has been reported in a heterologous expression system.³⁷ As shown in Fig. 3F, in unstimulated glands from fasted mice, Trpml1 was present in a serine phosphorylated form. However, up to 180 min following histamine stimulation, only a small fraction of Trpml1 was recovered in the phosphoserine IP, indicating that Trpml1 was dephosphorylated following parietal cell activation. Ezrin, a serine and tyrosine phosphorylated cytoskeletal protein involved in parietal cell activation⁹ served as a loading control.

Lysosomal Enlargement and Accumulation in Trpml1^{-/-} Mice

Histological analysis of gastric glands from 4 weeks old mice revealed a striking alteration of parietal cell morphology resulting from Trpml1 deletion. WT parietal cells are shown in Fig. 4A (asterisks). Notably, Trpml1^{-/-} parietal cells were grossly enlarged and contained numerous vacuoles that filled their cytoplasm (Fig. 4B, asterisks). Large multivesicular cytoplasmic inclusions present in every Trpml1^{-/-} parietal cell resulted in a distention of the glandular lumen (L, Fig. 4B). Remarkably, chief cells (CC, Fig. 4B) that were adjacent to affected parietal cells at the base of gastric pits were not vacuolated, supporting our findings of low Trpml1 expression in the lysosomes of these cells (Fig. 3A), and suggesting that the

pattern of Trpm1 expression is cell-type specific. IHC with lamp-1 antibody confirms the presence of markedly enlarged lysosomes in Trpm1^{-/-} parietal cells (Fig. 4C, asterisks) and contrasts with the sparse lysosomal staining seen in WT parietal cells (Fig. 4C, inset). To further characterize the morphological defects in Trpm1^{-/-} parietal cells, the gastric mucosa from WT and Trpm1^{-/-} littermates was subjected to electron microscopy. A representative micrograph of a resting WT parietal cell (Fig. 4D) shows the characteristic unstimulated parietal cell ultrastructure with numerous mitochondria (M), abundant cytoplasmic tubulovesicles (TV) and no visibly expanded secretory canaliculus. By contrast, the cytoplasm of Trpm1^{-/-} parietal cells displayed numerous individual and sometimes interconnecting single membrane bound vacuoles that contained multilamellated membrane whorls, characteristic of lysosomal storage diseases (arrows, Fig. 4E). In addition, resting Trpm1^{-/-} parietal cells displayed numerous dilated secretory canaliculi (SC, Fig. 4E) lined with elongated microvilli and no cytoplasmic tubulovesicles, consistent with a stimulated parietal cell configuration.³ Secretory canaliculi were present inside the cells and at the luminal membranes (L, Fig. 4E). The lysosomal accumulation in Trpm1^{-/-} parietal cells supports the previously proposed impaired endosomal/lysosomal biogenesis hypothesis for MLIV^{24, 38} and points to a blockade in the progression of lysosomal maturation that resulted in the prevalence of lysosomal transport intermediate organelles. We evaluated the genesis of lysosomal enlargement in Trpm1^{-/-} parietal cells by looking at the ultrastructure of the gastric mucosa from newborn WT and Trpm1^{-/-} mice. The ontogeny of gastric acid secretion in rodents described maturation in parietal cell acid secretory responses from birth up to about 2 weeks of age.³⁹ Numerous lysosomal inclusions were present in parietal cells from Trpm1^{-/-} neonatal mice (Supplementary Fig. 2 A–C), indicating that Trpm1 is essential to parietal cell function even before full maturation of acid secretion.

Stomach Hypertrophy and Hypergastrinemia in Trpm1^{-/-} Mice

The stomach mass of Trpm1^{-/-} mice was greater than WT littermates (Fig. 5A) and occupied an increased proportion of their total body weights (Fig. 5B, n = 10 – 12). In addition, Trpm1^{-/-} parietal cells were markedly enlarged (Fig. 5C, 10.56 ± 0.28 µm in WT versus 20.45 ± 0.95 µm in KO, n = 100 cells per group, p < 0.05), although parietal cell number per gland was not different between WT and Trpm1^{-/-} gastric glands (data not shown). Trpm1^{-/-} mice displayed elevated fasting serum gastrin levels at 8 weeks and markedly increased stomach gastrin mRNA (Fig. 5D and inset).

Defective Intracellular Canaliculi Formation in Trpm1^{-/-} Parietal Cells

As expected following histamine stimulation, WT parietal cells displayed well-developed, F-actin-rich intracellular canaliculi (Fig. 6A, arrows) and H⁺K⁺-ATPase was localized within this layer (Fig. 6 B and C). By contrast, in histamine stimulated Trpm1^{-/-} gastric glands, immunofluorescence for F-actin was present at the glandular luminal membranes (L, Fig. 6D), but was weak and diffuse within the parietal cells. Although H⁺K⁺-ATPase containing membranes in Trpm1^{-/-} cells stained diffusely for F-actin (Fig. 6 D – F, asterisks), a continuous actin-rich, intracellular canaliculi network was absent in stimulated Trpm1^{-/-} parietal cells. Since discrete secretory canaliculi were observed in Trpm1^{-/-} parietal cells by EM (Fig. 4E), the loss of F-actin staining on these membranes would suggest a defective secretory membrane formation/expansion.

Functional Association of Lysosomes with the Intracellular Canaliculi

Towards understanding the mechanisms underlying lysosomal involvement in intracellular canalicular formation and dynamics, we investigated a direct molecular interaction between H⁺K⁺-ATPase and Trpm1 in resting and stimulated gastric glands by coimmunoprecipitation (coIP). IP of Trpm1 from membrane extracts of control or histamine stimulated gastric glands, did not coIP H⁺K⁺-ATPase-α (Fig. 7A) or H⁺K⁺-

ATPase- β (not shown), indicating the lack of a direct interaction. Next, we measured lysosomal Ca^{2+} and receptor-mediated Ca^{2+} signaling in isolated WT and $\text{Trpm1}^{-/-}$ gastric glands. Lysosomal Ca^{2+} was measured using GPN which selectively bursts lysosomes and releases their Ca^{2+} content to the cytosol³⁰. The M3 receptors were then stimulated with carbachol to assay for receptor-mediated Ca^{2+} release and finally cells were exposed to Ca^{2+} -free and Ca^{2+} -containing media to assay for receptor-mediated Ca^{2+} influx. As shown in Fig. 7 (B–D), loss of Trpm1 with the consequent lysosomal accumulation had no effect on lysosomal Ca^{2+} storage or Ca^{2+} signaling ($n = 10$ WT and $n = 8$ $\text{Trpm1}^{-/-}$ glands from 4 and 3 mice, respectively), indicating that the effects of Trpm1 on intracellular canaliculi formation are not mediated through lysosomal Ca^{2+} or receptor-mediated Ca^{2+} signaling. Finally, we looked at the distribution of H^+K^+ -ATPase in relation to the expanded lysosomal compartment in $\text{Trpm1}^{-/-}$ parietal cells by confocal microscopy. Fig. 7E shows increased localization of H^+K^+ -ATPase on lamp-1 positive membranes in $\text{Trpm1}^{-/-}$ parietal cells (lower panel, asterisks) compared with WT parietal cells (top panel). The percent of colocalized intensity for H^+K^+ -ATPase and lamp-1 (calculated using ImarisColoc, Bitplane) was $28 \pm 5\%$ in $\text{Trpm1}^{-/-}$ and $3 \pm 0.2\%$ in WT parietal cells ($n = 10$ cells per group). The presence of proton pumps on the lysosomal membranes would suggest their mislocalization to this compartment in $\text{Trpm1}^{-/-}$ parietal cells and may explain the aggregated forms of H^+K^+ -ATPase α -subunit observed in $\text{Trpm1}^{-/-}$ stomach (Fig. 2D).

DISCUSSION

This study provides important insights into the loss of acid secretion observed in MLIV patients and implicates Trpm1 in the regulated membrane trafficking that is required for the translocation of H^+K^+ -ATPase between TVs and the apical canalicular secretory membranes.⁴ Trpm1 is highly expressed in gastric parietal cells where it is dephosphorylated and palmitoylated following stimulation of acid secretion. Trpm1 dephosphorylation could function to activate the channel and its palmitoylation could function in targeting Trpm1 to lipid microdomains on the apical membranes.

Similar to the achlorhydria in MLIV patients,^{10–11} basal and histamine-stimulated gastric acid secretion is markedly reduced in Trpm1 -deficient mice. The residual acid secretion in the $\text{Trpm1}^{-/-}$ mice could be due to a greater capability of mouse parietal cells to secrete acid in the presence of extensive lysosomal storage. In addition, unlike in the MLIV patients, direct measurement of acid secretion in the mice represents a true reflection of their secretory capacity. Nonetheless, $\text{Trpm1}^{-/-}$ parietal cells showed a marked reduction in mRNA and protein levels of H^+K^+ -ATPase α -subunit, formation of proton pump aggregates and localization of the proton pump to aberrant intracellular canalicular membranes. The marked reduction in the proton pump levels may have resulted from a feedback response caused by leakiness of gastric acid secretion into the aberrant intracellular canalicular. Future experiments will characterize the formation and nature of the H^+K^+ -ATPase α -subunit protein aggregates.

A striking ultrastructural defect present in unstimulated $\text{Trpm1}^{-/-}$ gastric glands is the prevalence of activated parietal cell ultrastructure with prominently expanded secretory canaliculi. Parietal cell activation could be secondary to the hypergastrinemia present in the $\text{Trpm1}^{-/-}$ mice³² or to an impaired ability to transition to the resting state and reestablish the cytoplasmic TV compartment. An *in vitro* assay of apical membrane recycling in $\text{Trpm1}^{-/-}$ parietal cells should shed more light into this mechanism. The presence of the activated phenotype did not, however, result in increased basal gastric acid secretion.

Ultrastructural analysis revealed that the $\text{Trpm1}^{-/-}$ parietal cell lysosomal inclusions were similar to previously described lysosomal storage bodies in MLIV cells and tissues

(heterogenous granular and multilamellated vacuoles¹⁷) except that the parietal cell inclusions were prominently enlarged and more numerous than in non-parietal cells. These inclusions were also present in parietal cells from neonatal *Trpml1*^{-/-} mice. The exaggerated susceptibility of parietal cells to *Trpml1* loss may be due to specialized functions that include an amplified apical membrane recycling system with high endocytic and lysosomal trafficking rates, and gastric acid secretion that may occur inside the cells within the trapped canaliculi, and perhaps inside lysosomes leading to the lysosomal swelling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ABE	acyl-biotinyl exchange
coIP	coimmunoprecipitation
IF	immunofluorescence
MLIV	mucopolipidosis type IV
SC	intracellular secretory canaliculus
TRPML1	transient receptor potential, mucolipin 1
TVs	tubulovesicles

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Figure 1.

Inactivation of the mouse *Trpm1* gene. (A) The mouse *Trpm1* genomic locus (top) and the vector (middle) designed to replace exons 1 to 3 of *Trpm1* resulting in the targeted *Trpm1* allele (bottom). (B) Southern blotting of Hind III-digested genomic DNA shows the 18-kb WT allele and the 15-kb recombinant allele in ES cells. (C) PCR genotyping of tail DNA from litters of *Trpm1*^{+/-} matings showing a 0.8-kb and a 0.5-kb fragment corresponding to the WT and targeted alleles, respectively. (D) Northern blot analysis shows a 2.5 kb *Trpm1* mRNA in WT and heterozygous mice and its loss in *Trpm1*^{-/-} mice brains. (E) Immunoblotting with anti-*Trpm1* C-terminus antibody shows the 65 kDa full length *Trpm1* in stomach and the cleaved 40 kDa C-terminal fragment in WT but not in *Trpm1*^{-/-} tissues.

Figure 2.

Impaired basal and histamine-stimulated acid secretion in $\text{Trpm1}^{-/-}$ mice. The baseline gastric pH is significantly higher in 16 weeks old Trpm1 -null mice ($-/-$) than WT ($+/+$) littermates (A). Trpm1 -deficient mice also secreted reduced amounts of gastric acid in response to histamine stimulation at 16 weeks (B) and at 8 and 24 weeks of age (C). Immunoblotting of H⁺K⁺-ATPase α - and β -subunit in 4 weeks old mice (D) shows reduced levels of the gastric proton pump and the presence of aggregated, high molecular weight forms of H⁺K⁺-ATPase α in $\text{Trpm1}^{-/-}$ mice stomachs (arrows). The data is representative of immunoblots from six littermates. Northern analysis (E) shows reduced H⁺K⁺-ATPase α levels in WT and $\text{Trpm1}^{-/-}$ gastric mucosa.

Figure 3.

Trpml1 is palmitoylated and dephosphorylated following histamine stimulation of acid secretion. (A) Confocal IF of frozen WT gastric glands stained with anti-Trpml1 antibody shows punctate cytoplasmic Trpml1 staining in parietal cells, which were identified with H^+K^+ -ATPase β - antibody (B). Trpml1 positive staining is also present on the luminal regions (arrows). The basolateral membrane staining in (B) is unrelated to H^+K^+ -ATPase β as it was present when the H^+K^+ -ATPase antibody was omitted. (C) Merged Trpml1 and H^+K^+ -ATPase β staining. (D) Trpml1 immunoblotting of input and ABE-enriched streptavidin eluate from WT and Trpml1^{-/-} glandular stomachs. Trpml1 is present in the ABE-enriched, hydroxylamine-treated WT extracts but not in Tris buffer-treated control. The input lanes contained 1% of the total protein used for the ABE assay. (E–F) WT and Trpml1^{-/-} littermates were injected with histamine for 40- or 180-min or without (control, C) and the stomach extracts were analyzed by ABE or anti-phosphoserine immunoprecipitation (IP). (E) Trpml1 palmitoylation is augmented 6-fold at 180 min following histamine stimulation of acid secretion. (F) Trpml1 is present in the phosphoserine immune complex in resting WT stomach but dephosphorylated upon histamine treatment. Snap-25 and Ezrin verified equal protein loading. The data is representative of three experiments. Scale bar = 25 μ m.

Figure 4.

Histopathological and ultrastructural changes in *Trpml1*^{-/-} gastric mucosa. Toluidine blue staining (A and B) and lamp-1 IHC (C) of gastric mucosa from 4 weeks old WT (A) and *Trpml1*^{-/-} (B and C) mice shows *Trpml1*^{-/-} parietal cells containing numerous vacuoles of varying sizes and shapes (asterisks, B and C). Chief cells (CC) were not vacuolated. Dilated glandular lumen (L) in *Trpml1*^{-/-} mice. The enlarged vacuoles stained strongly for lamp-1 confirming their lysosomal origin (C, inset shows comparable WT parietal cell staining). A representative electron micrograph of resting WT parietal cell (D) shows numerous tubulovesicles (TV), mitochondria (M) and no visibly expanded secretory canaliculi. By contrast, *Trpml1*^{-/-} parietal cells (E) displayed multivesicular and multilamellated vacuoles and expanded secretory canaliculi (SC) with elongated microvilli, both inside the cell and at the luminal membrane (L), characteristic of an activated parietal cell conformation. Scale bar = 20 μ m (A–C), 5 μ m (E).

Figure 5.

Stomach hypertrophy and hypergastrinemia in *Trpml1*^{-/-} mice. A representative photograph of whole stomach (A) shows increased stomach size in *Trpml1*^{-/-} mice which occupied an increased proportion of the total body weight (B). Parietal cell size is significantly increased in *Trpml1*-deficient gastric glands (C). Elevated serum gastrin is present in *Trpml1*^{-/-} mice (D) and Northern analysis shows increased gastrin mRNA levels in *Trpml1*^{-/-} stomachs (D, inset). Scale bar = 1 cm.

Figure 6.

Abnormal intracellular canalicular formation in parietal cells from *Trpml1*-deficient mice. Confocal IF shows the intracellular canalicular structure in histamine stimulated WT gastric glands (A–C), visualized by costaining for F-actin and H⁺K⁺-ATPase β -subunit. By contrast, F-actin staining is diffuse in histamine stimulated *Trpml1*^{-/-} parietal cells (D–F) and H⁺K⁺-ATPase containing membranes (asterisks) were only weakly stained for F-actin. Scale bar = 25 μ m.

Figure 7. Functional association of lysosomes with intracellular canaliculi formation. (A) IP of Trpm1 from stomach extracts did not coIP H⁺K⁺-ATPase α , indicating that Trpm1 did not directly interact with H⁺K⁺-ATPase in resting or histamine stimulated gastric glands. (B–D) Ratiometric imaging of intracellular Ca^{2+} in isolated WT and Trpm1 $^{-/-}$ gastric glands, showing that the loss of Trpm1 did not affect lysosomal Ca^{2+} store or receptor-mediated Ca^{2+} signaling. (E) Increased colocalization of H⁺K⁺-ATPase and lamp-1 positive lysosomal membranes (asterisks) in Trpm1 $^{-/-}$ parietal cells. Scale bar = 25 μ m.