**Drosophila** TRPML Forms PI(3,5)P₂-activated Cation Channels in Both Endolysosomes and Plasma Membrane*

Received for publication, July 30, 2013, and in revised form, December 11, 2013. Published, JBC Papers in Press, December 27, 2013, DOI 10.1074/jbc.M113.506501

**Xinghua Feng**, **Yu Huang**, **Yungang Lu**, **Jian Xiong**, **Ching-On Wong**, **Pu Yang**, **Jintang Xia**, **De Chen**, **Guangwei Du**, **Kartik Venkatachalam**, **Xuefeng Xia**, and **Michael X. Zhu**

From the 1Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, 510150, the 2Department of Integrative Biology and Pharmacology, 3Graduate Program in Cell and Regulatory Biology, and 4Graduate Program in Neuroscience, the University of Texas Health Science Center at Houston, Houston, Texas 77030, and the **Center for Genomic Medicine, Houston Methodist Research Institute, Houston, Texas 77030**

**Background:** *Drosophila* trpml mutants reproduced many defects associated with mucolipidosis type IV, but the fly TRPML channel remains uncharacterized.

**Results:** *Drosophila* TRPML is a phosphoinositide-regulated cation channel on endolysosome and plasma membranes.

**Conclusion:** Fly TRPML largely resembles mammalian TRPML1, but exhibits differences in subcellular localization and pH dependence.

**Significance:** The data support using *Drosophila* for assessing TRPML1 function.

Transient Receptor Potential mucolipin (TRPML) channels are implicated in endolysosomal trafficking, lysosomal Ca²⁺ and Fe²⁺ release, lysosomal biogenesis, and autophagy. Mutations in human TRPML cause the lysosome storage disease, mucolipidosis type IV (MLIV). Unlike vertebrates, which express three TRPML genes, TRPML1–3, the *Drosophila* genome encodes a single trpml gene. Although the trpml-deficient flies exhibit cellular defects similar to those in mammalian TRPML1 mutants, the biophysical properties of *Drosophila* TRPML channel remained uncharacterized. Here, we show that transgenic expression of human TRPML in the neurons of *Drosophila* trpml mutants partially suppressed the pupal lethality phenotype. When expressed in HEK293 cells, *Drosophila* TRPML was localized in both endolysosomes and plasma membrane and was activated by phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) applied to the cytoplasmic side in whole lysosomes and inside-out patches excised from plasma membrane. The PI(3,5)P₂-evoked currents were blocked by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) but not other phosphoinositides. Using TRPML A487P, which mimics the varitint-waddler (Va) mutant of mouse TRPML3 with constitutive whole-cell currents, we show that TRPML is biphasically regulated by extracytosolic pH, with an optimal pH about 0.6 pH unit higher than that of human TRPML1. In addition to monovalent cations, TRPML exhibits high permeability to Ca²⁺, Mn²⁺, and Fe²⁺, but not Fe³⁺. The TRPML currents were inhibited by trivalent cations Fe³⁺, La³⁺, and Gd³⁺. These features resemble more closely to mammalian TRPML1 than TRPML2 and TRPML3, but with some obvious differences. Together, our data support the use of *Drosophila* for assessing functional significance of TRPML1 in cell physiology.

In vertebrates, the mucolipin subfamily of Transient Receptor Potential mucolipin (TRPML) proteins is comprised of three members: TRPML1, TRPML2, and TRPML3 (MCOLN1–3 for gene names) (1). Mutations of TRPML1 in human are linked to a lysosomal storage disease known as mucolipidosis type IV (MLIV), characterized by severe neurodevelopmental delay, progressive vision loss, neurodegeneration, motor defects, and iron-deficiency anemia (2–5). TRPML1 is widely expressed in many cells and it forms non-selective Ca²⁺-permeable cation channels primarily localized in the late endosomes and lysosomes (1). Cells from MLIV patients display abnormal accumulation of lipids in endolysosomes, enlarged vacuoles (6), and abnormal vesicle trafficking (7). The physiological and cellular defects of MLIV were largely reproduced in mice with the ablation of MCOLN1 gene (8–10), confirming the critical role of TRPML1 in endolysosomal trafficking and its contribution to MLIV.

Compared with TRPML1, less is known about the roles of TRPML2 and TRPML3 in endolysosomal trafficking. The cell type distributions for these two isoforms are also more restricted than that of TRPML1 (1). These two TRPML isoforms have, in general, a broader subcellular distribution than TRPML1 and they are found to be present on the plasma membrane (11, 12). The plasma membrane TRPML2 and TRPML3 channel activities appear to be inhibited by extracellular Na⁺, as removing Na⁺ from the extracellular solution allowed development of the constitutive TRPML2 and TRPML3 currents (12, 13). In addition, the Na⁺ inhibition is further augmented by acidic pH at the extracytosolic side (14, 15). Interestingly, a gain-of-function mutation of TRPML3 (A419P) associated

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*This work was supported in part by Grants R01 GM081658 and GM092759 from the National Institutes of Health (to M. X. Z.), postdoctoral fellowships from the Third Affiliated Hospital of Guangzhou Medical University (to X. F. and Y. L.), and the National Natural Science Foundation of China (No.81270868, to X. X.).

1 To whom correspondence may be addressed: The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China, 510150. E-mail: xuefengx@gmail.com

2 To whom correspondence may be addressed: Department of Integrative Biology and Pharmacology, The University of Texas Health Science Center at Houston, MSB 4.128, 6431 Fannin St., Houston, Texas 77030. Tel: 713-500-7505; Fax: 713-500-7444; E-mail: michael.x.zhu@uth.tmc.edu

3 The abbreviations used are: TRPML, transient receptor potential mucolipin; MLIV, mucolipidosis type IV; PEI, polyethylenimine.
with the varitint-waddler (Va) phenotype lacks the Na⁺ inhibition and shows constitutive inwardly rectifying cation currents on the plasma membrane (13, 16–18). The Va mice show defects in hearing and pigmentation, implicating a key function of TRPML3 in hair cells and melanocytes (19). Substitutions of the equivalent position, as well as a number of nearby amino acids, by proline in TRPML1 or TRPML2 also resulted in constitutive channel activity on the plasma membrane (16, 17, 20). These have allowed convenient characterizations of biophysical properties of these channels (21).

Common to all mammalian TRPML channels is their activation on the cytoplasmic side by phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂), a phosphoinositol enriched in intracellular membranes (22). At least for TRPML1, this effect appears to be antagonized by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) (23), the PIP₂ species enriched in the plasma membrane. Thus, it may be assumed that selective bindings between PI(3,5)P₂ and PI(4,5)P₂ determine the function of TRPML channels. While the high PI(4,5)P₂ content inhibits the TRPML channels located on the plasma membrane, the high PI(3,5)P₂ levels in intracellular membranes support the activation of TRPML channels in endolysosomes.

Different from vertebrates, Drosophila melanogaster and Caenorhabditis elegans express only one trpml gene. In C. elegans, the deletion of the MCOLN ortholog gene, cup-5, leads to the accumulation of enlarged vacuoles, excess apoptotic cells, and embryonic lethality (24, 25). In Drosophila, disruption of the only trpml gene resulted in similar, but more severe, defects as compared with human MLIV, including defective autophagy due to diminished fusion of late-endosomes and amphisomes with lysosomes, impaired synaptic transmission, accumulation of apoptotic cells, oxidative stress, lipofuscin accumulation, mitochondrial dysfunction, motor defects, and massive neurodegeneration (1, 26–28). The more severe abnormality of the Drosophila TRPML knock-out than the mammalian TRPML1 knock-out/mutations suggests that the insect channel may also carry out functions that are uniquely performed by mammalian TRPML2 and/or TRPML3, raising the possibility that the insect TRPML channel may collectively possess properties of all three mammalian TRPML channels. To date, however, little is known about the channel function of Drosophila TRPML channel and how it is regulated.

Here, we show that transgenic expression of human TRPML1 in neurons of the TRPML-null flies (referred to as trpml⁻¹ (26)) partially rescued the pupal lethality phenotype, indicating some but not complete functional resemblance between the insect TRPML and mammalian TRPML1. To provide functional correlates between these channels, we examined the channel function of Drosophila TRPML heterologously expressed in HEK293 cells. We show that TRPML is expressed and functional on both endolysosomes and plasma membrane of HEK293 cells. Similar to its mammalian counterparts, it is activated by PI(3,5)P₂ and inhibited by PI(4,5)P₂. However, its sensitivity to other phosphoinositides is not identical to that of mouse TRPML1. The “Va” mutant of Drosophila TRPML is constitutively active on the plasma membrane and is permeable to K⁺, Na⁺, Ca²⁺, Mn²⁺, and Fe²⁺, but blocked by trivalent cations, Fe³⁺ and La³⁺ and Gd³⁺. In addition, the channel displays a bell-shaped dependence on extracytosolic pH, which is significantly left shifted as compared with that of human TRPML1.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Compounds**—The construct for C-terminal YFP-tagged Drosophila TRPML (26) was used to create the TRPML-EGFP plasmid transferring the coding sequence of TRPML to the pEGFP-N1 vector (Clontech). The Lamp1-mCherry plasmid was generated from Lamp1-EGFP (gift from Dr. Paul Luzio, Cambridge University) by replacing the coding sequence of EGFP with that of mCherry. Human TRPML1Va-EGFP was a gift from Dr. Haoxing Xu (University of Michigan). TRPMLVa-EGFP was created by substituting the codon for Ala487 of TRPML-EGFP by that for a proline using the QuikChange Site-directed Mutagenesis kit (Stratagene). The final sequence was verified by DNA sequencing.

All phosphoinositides (Di-C8 forms, unless indicated otherwise) were purchased from Cayman Chemical Co. Other chemicals were obtained from Fisher Scientific Co or Sigma-Aldrich unless indicated otherwise. Stock solutions of diC16 phospholipids were made in water (0.5 mM) by sonication for 30 min on ice and then aliquots stored at −80 °C. On the day of the experiments, a new aliquot was thawed and diluted in the bath solution for inside-out recordings (see later under “Electrophysiology”) and sonicated for 10–30 min. This procedure followed that described previously for PI(4,5)P₂ (29), allowing the formation of mostly small micelles for channel activation in the patched membrane.

**Transgenic Flies and Pupal Lethality Analysis**—The cDNA for C-terminal hemagglutinin (HA)-tagged human TRPML1 (hTRPML1::HA) was subcloned into the pUAST vector and transgenic flies were generated by Rainbow Transgenic Flies Inc. To verify transgene expression in Drosophila cells, the flies were crossed with fly line cg-GAL4 (Bloomington line 7011) for expression in fat-bodies/hemocytes and the presence of hTRPML1::HA in larval fat-bodies was detected by immunohistochemistry as described (27). Briefly, fat-bodies were dissected from 3rd instar wandering larvae and incubated in phosphate-buffered saline (PBS) containing LysoTracker Red DND-99 (1:500) (Invitrogen) at room temperature for 30 min. The samples were then washed two times with PBS, and fixed in 4% paraformaldehyde in PBS for 30 min. Samples were then washed three times with PBS plus 0.01% Triton X-100 before incubating with rabbit anti-HA primary antibodies (Cell Signaling) at 4 °C overnight. The tissues were then washed and probed with fluorophore-conjugated secondary antibodies (Alexa Fluor 488 goat antirabbit (Invitrogen) at room temperature for 1.5 h. Samples were mounted on glass slide with Vectashield Mounting Media containing DAPI (Vector Labs). Images were taken using a Nikon A1 confocal microscope system.

For pupal lethality analysis, hTRPML1::HA was expressed in the trpml⁻¹ neurons using a pan-neuronal GAL4 driver (elav-GAL4) as described (26). The numbers of eclosed pupal cases and uneclosed, or partially eclosed, pupae after metamorphosis were counted to determine pupal lethality.

**Cell Culture and Transfection**—HEK293 cells were cultured as previously described (30). Transfections were performed in
Functional Characterization of Drosophila TRPML

wells of a 96-well plate using polyethylenimine (PEI) (31, 32). Briefly, cells were grown overnight in wells of a 12-well plate to 40–50% confluence. The desired plasmid (0.5 μg) was diluted in 50 μl normal saline (150 mM NaCl, sterile) and then mixed with 4 μl of deacetylated PEI (25 mM) by vortexing. After incubation at the room temperature for 10 min, the DNA/PEI mixture was added dropwise to the cultured cells while the plate was gently rocked throughout the process to allow even distribution of the DNA/PEI complex. The plate was then returned to the culture incubator. The cells were reseeded 16–20 h later on poly-ornithine coated glass coverslips and used within 4–24 h for fluorescence imaging or electrophysiological studies.

Fluorescence Imaging—Cells co-transfected with TRPML-EGFP and Lamp1-mCherry grown on glass coverslips were fixed by 4% paraformaldehyde in PBS for 20 min at room temperature and rinsed in PBS. The coverslip was mounted on a glass slide using fluorogel (with Tris buffer) mounting media (Electron Microscopy Sciences). Fluorescence images were taken using a Nikon eclipse Ti laser scanning confocal microscope system. To document the enlarged endolysosomal vacuoles, cells transiently transfected with TRPML-EGFP grown on glass coverslips were treated overnight with vacuolin-1 (1 μM). DIC and epifluorescence images were taken using a 63× water-immersed lens from an upright Olympus BX51WI fluorescence microscope.

Electrophysiology—For whole-cell recordings, the pipette solution contained (in mM): 135 Cs-methanesulfonate, 10 CsCl, 1 MgCl₂, 1 EGTA, 4 HEPES (pH 7.2 by CsOH). Recording pipettes were pulled from standard wall borosilicate tubing with filament (Sutter Instrument) to 2–3 MΩ when filled with the pipette solution and placed in the bath. The normal Tyrode’s bath solution contained (in mM): 135 NaCl, 5.6 KCl, 2.6 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 by NaOH). The low pH bath solution contained (in mM): 135 NaCl, 5.6 KCl, 2.6 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, and 10 2-morpholinoethanesulfonic acid (MES), (pH 4.6 or other desired pH values by HCl or NaOH). The N-methyl-d-glucamine (NMDG) solution contained (in mM): 140 NMDG, 10 glucose, 10 HEPES (pH 7.4 by HCl). The low sodium bath solution (LSS) contained (in mM): 150 KCl, 2 NaCl, 0.25 CaCl₂, 10 glucose, 10 HEPES (pH 7.4 by KOH). For ion selectivity experiments, the isotonic extracellular solution contained (in mM): 150 NaCl, 150 KCl, 150 CsCl, 100 CaCl₂, or 100 MnCl₂, supplemented with 10 HEPES and 10 MES (pH 4.6 by HCl). For experiments testing permeability of Fe²⁺ and Fe³⁺, the extracellular Fe²⁺ or Fe³⁺ solution contained (in mM) 50 FeCl₂ or 35 FeCl₃, supplemented with 10 HEPES and 10 MES and the required amount of NMDG to reach an osmolarity of 300 mosm (pH 4.6 by HCl). Ascorbic acid (50 μM) was included in the Fe³⁺ solution to prevent oxidation.

For whole lysosome patch clamp recordings, cells transfected with TRPML-EGFP were treated with vacuolin-1 (1 μM) overnight and then placed in the recording chamber mounted on an Olympus IX71-inverted fluorescence microscope. A sharp glass pipette mounted to a micromanipulator was used to slice through a selected EGFP-positive cell that contained enlarged green fluorescence vacuoles (see Fig. 2B). One of the vacuoles released from the cell was then picked for whole lysosome recording using a patch electrode filled with the low pH bath solution (pH 4.6) described above. For both whole lysosome and inside-out recordings, the bath solution contained (in mM): 140 K-glucosate, 4 NaCl, 2 MgCl₂, 0.39 CaCl₂, 1 EGTA, 10 HEPES (pH 7.2 by KOH). The pipette for inside-out recordings contained the normal Tyrode’s solution. The pipette resistance was 2–3 MΩ for inside-out patches and 4–5 MΩ for whole-lysosome recordings. The capacitance of the whole lysosomes used for recording was around 1 pF.

Drugs (phosphoinositides and trivalent cations) were diluted to the final desired concentrations in the appropriate solutions and were applied via a gravity-driven perfusion system with the open end placed at about 50 μm from the sample being recorded. The solution exchange was complete within a few seconds.

Data were collected by an EPC9 or EPC10 amplifier using PatchMaster (HEKA electronics Inc., Germany). Cells, patches, or enlarged endolysosomes, were held at 0 mV. Currents were elicited by voltage ramps from −100 to +100 mV in 200 ms and repeated every second. For voltage steps in Fig. 3C, 1 s steps from −100 to 90 mV in 10-mV increments and with 2-s intervals were used; for voltage steps in Fig. 5D, 400 ms steps from −80 mV to +80 mV in 10-mV increments and with 1-s intervals were used. Signals were digitized at 5 kHz and filtered at 2 kHz. All experiments were conducted at the room temperature (22–23°C).

Data Analysis—All data were analyzed and plotted using Origin 7.5 (OriginLab, Northampton, MA). Permeability ratios for monovalent cations were determined using the formula: $P_X/P_K = \exp(\Delta F_{\text{rev}}F/RT)$, where $P_X$ and $P_K$ are permeabilities of the cation X and K⁺, respectively, $\Delta F_{\text{rev}}$ is the difference between the reversal potential with cation X and that with K⁺. F, R, and T are Faraday constant, gas constant, and absolute temperature, respectively. Permeability ratios for divalent cations were calculated using the formula: $P_{XX}/P_{K^+} = (37.5/[X]_i) \times \exp(\Delta F_{\text{rev}}F/RT)/(\exp(\Delta F_{\text{rev}}F/RT) + 1)$, where [X]ᵢ represents concentration of the divalent ion in the bath solution and other symbols are the same as defined above. Summary data are presented as the mean ± S.E. Statistical comparisons were made using Student’s t test. A p value of < 0.05 was considered statistically significant.

RESULTS

Partial Rescue of Pupal Lethality Exhibited by Drosophila trpml-null Mutants Following Neuronal Expression of Human TRPML1—To gain insights on functional correlation between insect TRPML and mammalian TRPML1, we created a transgenic line expressing human TRPML1 in Drosophila using the PhiC31 integration system (33). To help confirm the expression in Drosophila cells, an HA tag was added to the C terminus of human TRPML1 (UAS-hTRPML1:HA), and the transgene was first introduced into fat bodies of wild type flies using cg-GAL4 because this cell type is enriched in endolysosomes and known to express TRPML (27). Immunostaining using an anti-HA antibody revealed strong fluorescence signals surrounding intracellular vesicles labeled by Lysotracker only in fat bodies dissected from larvae expressing hTRPML1:HA but not in tissues dissected from control flies (Fig. 1A), demonstrating that...
Functional Characterization of Drosophila TRPML

hTRPML1::HA can be expressed in insect cells and properly localized to acidic organelles. The Drosophila trpml1 mutants exhibit reduced viability during the pupal phase of metamorphosis, and this pupal lethality is fully rescued by pan-neuronal expression of UAS-trpml1 (26). To examine whether hTRPML1 could mimic the function of TRPML, we directed UAS-hTRPML1::HA into all neurons of larval fat-bodies from transgenic flies with neuronal expression of human TRPML1. confocal fluorescence images show LysoTracker Red DND-99 (violet) and Alexa Fluor 488 (green) stained acidic organelles and hTRPML1::HA, respectively. Scale bar, 10 μm and is the same for all panels. B, images of closed pupal case and unclosed (dead) pupa. C, pupal lethality of trpml1 mutants without and with pan-neuronal expression of UAS-hTRPML1::HA driven by elav-GAL4. n = 4 independent crosses. *, p < 0.001 versus trpml1.

Functional Expression of Drosophila TRPML in Endolysosomes and Plasma Membrane of HEK293 Cells—To study the channel function of TRPML, we expressed the C-terminal EGFP-tagged TRPML in HEK293 cells. This system was chosen because it has been most commonly used for functional studies of mammalian TRPML channels (12–23), providing basis for direct comparison between these channels. As demonstrated previously (26), the expressed EGFP fusion protein was mostly found in intracellular vesicles positively labeled by the late-en}

FIGURE 1. Partial rescue of pupal lethality of Drosophila trpml1 mutant by neuronal expression of human TRPML1. A, immunohistochemical staining by anti-HA antibody of larval fat-bodies from transgenic flies with UAS-hTRPML1::HA expression driven by the elav-GAL4 driver (upper panels) and control flies in which the UAS-hTRPML1::HA transgene was not expressed (lower panels). Confocal fluorescence images show LysoTracker Red DND-99 (violet) and Alexa Fluor 488 (green) stained acidic organelles and hTRPML1::HA, respectively. Scale bar, 10 μm and is the same for all panels. B, images of closed pupal case and unclosed (dead) pupa. C, pupal lethality of trpml1 mutant without and with pan-neuronal expression of UAS-hTRPML1::HA driven by elav-GAL4. n = 4 independent crosses. *, p < 0.001 versus trpml1.
Functional Characterization of Drosophila TRPML

In addition to the intracellular localization, obvious EGFP signal was also observed in the peripheral regions of the TRPML-EGFP transfected cells (Fig. 2A, arrow), suggesting that a fraction of the expressed proteins might be on the plasma membrane. This differs from mammalian TRPML1, which is almost extensively localized to endolysosomes (21, 22), but resembles TRPML2 and TRPML3, which are present on plasma membrane (11–13). To examine if functional channels were formed on the plasma membrane by TRPML-EGFP, we recorded ionic currents by voltage clamp recordings. No obvious current was detected from transfected cells under the whole-cell configuration, which allows application of drugs from the cytoplasmic side. Micropatches were excised from the plasma membrane by TRPML-EGFP, we recorded current from excised I/O patches. Note the slow rise of current elicited by diC8 PI(3,5)P2. The above experiments used diC8 PI(3,5)P2, which contains shorter acyl chains than the physiological phospholipids. To test if the full-length PI(3,5)P2 could also activate TRPML, we applied diC16 PI(3,5)P2, to the I/O patches after the response to 1 μM diC8 PI(3,5)P2 had become stable. At 1 μM, diC16 PI(3,5)P2 did not consistently elicit a response, but at 3 and 10 μM, it induced TRPML current with a similar current-voltage (I-V) relationship as that evoked by diC8 PI(3,5)P2 (Fig. 3E and F). Noticeably, the current developed more slowly in diC16 than in diC8 PI(3,5)P2. The time to reach half-maximal amplification of the PI(3,5)P2-evoked current in inside-out patches excised from plasma membrane of HEK293 cells expressing Drosophila TRPML. A, representative time courses of currents at −100 mV and +100 mV recorded using voltage ramps from an inside-out (I/O) patch elicited by TRPML-transfected cell. diC8 PI(3,5)P2 (1 μM) was bath applied multiple times as indicated to the cytoplasmic side. Note the time-dependent run-up of the PI(3,5)P2-evoked currents. B, I-V curves obtained by voltage ramps at the indicated time points in A. C, representative current traces for the same patch as shown in A elicited by voltage steps (−100 to +90 mV, with 10 mV increments, a step duration of 1 s and an interval of 2 s between consecutive steps) from the holding potential of 0 mV in the absence (upper) and presence (lower) of 1 μM diC8 PI(3,5)P2. D, concentration dependence of diC8 PI(3,5)P2-evoked currents from inside-out patches. Currents were measured using the similar protocol as in A, with varying concentrations of diC8 PI(3,5)P2 after the currents elicited by 1 μM diC8 PI(3,5)P2 had stabilized. Current amplitudes at −100 mV were normalized to that elicited by 1 μM diC8 PI(3,5)P2. Data points (means ± S.E., n = 7) were fitted with the Hill equation, which yielded an EC50 of 0.39 ± 0.07 μM. E, similar to A, but with consecutive application of diC8 PI(3,5)P2 and diC16 PI(3,5)P2 (both at 3 μM) as indicated. Note the slow rise of current elicited by diC16 PI(3,5)P2. F, I-V curves obtained by voltage ramps at the indicated time points in E.
Inhibited the currents activated by PI(3,5)P2 in a concentration-dependent manner. Therefore, we tested whether PI(4,5)P2 could inhibit the currents activated by PI(3,5)P2 in I/O patches. The slower activation kinetics has also been reported for currents of plasma membrane localized K+ channels activated by PI(4,5)P2 of long acyl chains than shorter ones (29, 34), presumably because of micelle formation. In subsequent experiments, we only used the diC8 forms of phosphoinositides. 

Inhibition of TRPML by PI(4,5)P2.—The run-up of PI(3,5)P2-induced currents in I/O patches is consistent with the observation that mammalian TRPML is inhibited by PI(4,5)P2, which is enriched on the plasma membrane and slowly washed out under the I/O patch configuration in the absence of ATP (23). Therefore, we tested whether PI(4,5)P2 could inhibit the TRPML currents. Although very similar in structure, PI(4,5)P2 (1 μM) did not evoke the TRPML current from I/O patches like PI(3,5)P2 did when applied alone (Fig. 4, A and B). Instead, it inhibited the currents activated by PI(3,5)P2 in a concentration-dependent manner (Fig. 4, C–E). At 3 μM PI(4,5)P2, which is lower than the resting plasma membrane PI(4,5)P2 concentration of 4–10 μM (23), the current evoked by PI(3,5)P2 (0.3 μM) at −100 mV was inhibited by about 75% (73.8 ± 1.1%, n = 4). The IC50 for PI(4,5)P2 inhibition was estimated to be 1.21 μM (n = 4).

To gain information about selectivity of TRPML among different phosphoinositides, we tested the effect of PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P2, and PI(3,4,5)P3 (all diC8) on PI(3,5)P2-induced current in the I/O patches excised from cells that expressed TRPML-EGFP. At 1 μM, none of the phosphoinositides significantly changed the basal current in the I/O patches, in which robust currents were elicited by PI(3,5)P2 (data not shown), suggesting that activation of TRPML is specifically dependent on PI(3,5)P2. We then used 10-fold excess of the phosphoinositi de concentration (3 μM) to test the effect on current elicited by PI(3,5)P2 (0.3 μM). Only PI(5)P caused significant, but small (15 ± 2%), inhibition, while PI(3,4)P2 and PI(3,4,5)P3 resulted in small (~10%), but significant, increase of the current (Fig. 4F). This profile is different from that of mouse TRPML1, for which PI(3,4)P2 and PI(3,4,5)P3 were equally potent as PI(4,5)P2 at inhibiting the current evoked by PI(3,5)P2 (23).

pH Dependence of TRPML.—The A419P mutation of mouse TRPML3 (Va mutant) led to constitutive channel activation and accumulation of the mutant channels on the plasma membrane. Equivalent mutations, but with V→P substitution for mammalian TRPML1 and A→P for TRPML2 also caused the similar effect (16, 17). Based on the alignment with mammalian TRPMLs (Fig. 5A), we mutated A487 of TRPML to a proline (A487P) to create TRPML1Va. As expected, expression of TRPML1Va in HEK293 cells led to constitutive inwardly rectifying...
Functional Characterization of Drosophila TRPML

**FIGURE 5.** Whole-cell currents of Drosophila TRPML<sup>Va</sup> expressed in HEK293 cells and its dependence on extracytosolic pH. A, alignment of putative transmembrane segment 5 of Drosophila TRPML with that of mouse TRPML1, TRPML2, and TRPML3. Names in parentheses are Gene Product ID in UniProtKB database. The amino acids mutated in the Va mutants are boxed and shaded in gray. The asterisk indicates Ala-487 of Drosophila TRPML, which was replaced with a proline in TRPML<sup>Va</sup>. B, summary (means ± S.E.) of current amplitudes at −100 mV from whole-cell recordings of basal activity in normal Tyrode’s bath solution by voltage ramps in HEK293 cells expressing TRPML<sup>Va</sup> or untransfected cells. C, representative I-V curves obtained by voltage ramps of whole-cell recording of an HEK293 cell expressing TRPML<sup>Va</sup> in normal Tyrode’s bath solution (basal), NMDG bath, and pH 4.6 Tyrode’s bath. W/C, whole-cell; D, representative traces of whole-cell currents elicited by voltage steps (−80 mV to +80 mV, with 10-mV increments and a step duration of 400 ms) from the holding potential of 0 mV in an HEK293 cell expressing TRPML<sup>Va</sup> in normal Tyrode’s bath (basal), left), NMDG (middle), and pH 4.6 Tyrode’s (right) bath solutions. E, representative time courses of currents at −100 mV and +100 mV recorded using voltage ramps by whole-cell recording of an HEK293 cell expressing TRPML<sup>Va</sup>. Constitutive basal currents were seen in normal Tyrode’s bath. NMDG and Tyrode’s solutions with different pH were applied as indicated through perfusion. F, I-V curves obtained by voltage ramps for selected conditions shown in E. G, summary of pH-dependent modifications of TRPML<sup>Va</sup> and human TRPML1<sup>Va</sup> (hTRPML1<sup>Va</sup>). Currents at −100 mV were normalized to that recorded in pH 5.0 Tyrode’s bath. Data points (means ± S.E., n = 5 for each) were fitted with Gaussian function.

ing currents readily detectable upon formation of the whole-cell configuration (Fig. 5, B and C). The inward rectification and reversal potential at near-0 mV were in agreement with the I-V relationship recorded for wild type TRPML from whole-lysosomes and I/O plasma membrane patches. The inward current of TRPML<sup>Va</sup> was eliminated by substitution of all cations in the Tyrode’s bath solution with NMDG (Fig. 5C), demonstrating cation selectivity. In response to voltage steps, the constitutive currents showed persistent currents at negative potentials with no obvious inactivation in 400 ms (Fig. 5D, left). These currents were again eliminated by the NMDG bath solution (Fig. 5D, middle). Therefore, the TRPML<sup>Va</sup> mutant displays constitutive activity on the plasma membrane without an obvious change in the biophysical properties. It is thus a suitable construct for further characterizations of TRPML properties when solution exchanges needed to be carried out from the extracellular side (equivalent to lysosome lumen). Similar approaches have been used to study the properties of mammalian TRPML channels (15, 16, 21).

It was previously shown that extracellular acidification (pH 4.6) enhanced the current of mouse TRPML1<sup>Va</sup> but reduced that of TRPML3<sup>Va</sup> (16). As a lysosomal channel, TRPML is also exposed to the acidic pH of the endolysosomal lumen. Therefore, it was important to know how its activity was affected by pH changes. Similar to mouse TRPML1<sup>Va</sup>, lowering extracellular pH from pH 7.4 to pH 4.6 markedly increased the TRPML<sup>Va</sup> currents by about 2-fold (Fig. 5, C and D, right). To further define the pH dependence of TRPML, we exposed the TRPML<sup>Va</sup>-expressing cells to a range of extracellular pH values, from pH 3 to 8 (Fig. 5E). Interestingly, the pH dependence is bell-shaped, with current peaked in between pH 5 and 6 and declined at either higher or lower pH values (Fig. 5E, F). Fitting normalized currents at −100 mV in different extracellular pH solutions with a Gaussian function yielded an optimal pH of 5.17 ± 0.04 for TRPML<sup>Va</sup>, which is about 0.6 pH unit less acidic than that of human TRPML1<sup>Va</sup> (4.45 ± 0.07), determined under the same conditions. Therefore, TRPML activity is potentiated by acidic pH commonly found in the lumen of endolysosomes but inhibited by strong acid. Compared with mammalian TRPML1, the pH dependence of TRPML is shifted to milder acidity.

**Ion Selectivity of TRPML**—Elimination of the inward currents by NMDG suggested that TRPML<sup>Va</sup> mediated cation currents (Figs. 5, C and D, and 6, A and B). To determine the ion selectivity of TRPML<sup>Va</sup>, we replaced the Tyrode’s bath solution with isotonic KCl solutions of different cations in the whole-cell configuration (Fig. 6A). The divalent-free (DVF) KCl solution caused an immediate increase in current amplitudes from that in the Tyrode’s solution at all potentials and then quickly reduced to an intermediate level. Switching to a DVF NaCl solution caused a further increase of the current amplitudes, which was strongly reduced by exchanging for a DVF CsCl solution (Fig. 6A). The relative currents of K<sup>+</sup> and Cs<sup>+</sup> to...
that of Na⁺ at −100 mV were 0.70 ± 0.4 (n = 5) and 0.13 ± 0.01 (n = 6), respectively (Fig. 6D). The permeability ratios of Na⁺/K⁺ and Cs⁺/K⁺ were 1.00 ± 0.06 (n = 3) and 1.00 ± 0.02 (n = 7), respectively, based the calculation using reversal potential values, suggesting about equal permeability to Na⁺, K⁺, and Cs⁻ for the heterologously expressed TRPML₁α channel.

We also tested the permeability to Ca²⁺ and Fe²⁺, both have been shown to permeate mouse TRPML1 (16, 21). Switching the extracellular solution to 100 mM CaCl₂ or 50 mM FeCl₂ markedly reduced the inward currents as compared with that in DVF isotonic NaCl, but the reversal potentials were changed to more positive than zero mV (Fig. 6, B and C), indicating that TRPML₁α had higher permeabilities for Ca²⁺ and Fe²⁺ than monovalent cations. The relative currents of Ca²⁺ and Fe²⁺ to that of Na⁺ at −100 mV were 0.23 ± 0.04 (n = 5) and 0.32 ± 0.03 (n = 3), respectively (Fig. 6D). The permeability ratios of Ca²⁺/Na⁺ and Fe²⁺/Na⁺ were 5.5 ± 1.2 (n = 8) and 12.0 ± 2.0 (n = 5), respectively, based the calculation using reversal potential values. The expressed TRPML₁α channel also showed permeability to Mn²⁺. The relative current of Mn²⁺ to that of Na⁺ at −100 mV was 0.24 ± 0.03 (n = 6) (Fig. 6D) and the permeability ratio of Mn²⁺/Na⁺ was 6.0 ± 1.8 (n = 5).

**Inhibition of TRPML by Trivalent Cations**—In contrast to Fe²⁺, TRPML₁α was impermeable to Fe³⁺. With the bath solution containing 35 mM FeCl₃, nearly no inward current was detectable, but the current recovered completely upon washout by 50 mM FeCl₂ (Fig. 6E). Not only was Fe³⁺ not able to permeate TRPML₁α, but also it inhibited the cation conductance of TRPML₁α in a concentration dependent manner (Fig. 7, A and B). In the pH 4.6 Tyrode’s solution, addition of 1 mM FeCl₃ inhibited the current at −100 mV by 36.2 ± 1.0% (n = 3) and 10 mM FeCl₃ inhibited the current by 72.5 ± 2.6% (n = 3).

Lanthanides, La³⁺ and Gd³⁺, have been frequently found to block non-selective cation channels, including many TRP channels (35). Including 1 mM La³⁺ or Gd³⁺ in the pH 7.2 Tyrode’s solution blocked the TRPML₁α current at −100 mV by 78 ± 2 or 74 ± 3%, respectively (Fig. 7, C, D, G). These levels of inhibition were similar to the inhibition of human TRPML₁α by the lanthanides (Fig. 7G). Interestingly, the degree of inhibition by both lanthanides was comprised in acidic extracellular pH and the effect was more pronounced for La³⁺ than Gd³⁺. At pH 4.6, 1 mM La³⁺ inhibited the TRPML₁α current by 35 ± 3%, and 1 mM Gd³⁺ inhibited it by 55 ± 3% (Fig. 7, E–G). These data suggest that like mammalian TRPML1, TRPML is inhibited by lanthanides and the inhibition is weakened by acidic pH commonly found in the lysosome lumen.

**DISCUSSION**

Unlike mammals, the fruit fly Drosophila melanogaster expresses only one TRPML isoform (26). Nonetheless, ablation of the Drosophila trpml gene in flies reproduced many of the cellular defects found in MLIV human patients and in MCOLN1 knock-out mice (26, 27), suggesting conserved functions in cellular physiology by fly TRPML and mammalian TRPML1. However, the lack of TRPML2 and TRPML3 would suggest that TRPML might also carry functions normally performed by these two TRPML isoforms in mammalian cells, which could help explain some of the more severe cellular abnormalities found in the Drosophila mutant than mammalian TRPML1 mutants. Indeed, we found that transgenic expression of human TRPML1 in neurons of Drosophila trpml-null mutant partially rescued the pupal lethality phenotype, demonstrating the conserved function between TRPML1 and TRPML. However, unlike the fly TRPML, which completely rescued pupal lethality when expressed using the same pan-neuronal promoter (26), the rescue by the human channel was only about 50%, indicating also significant functional divergence between the two channels. Thus, functional characterization of TRPML is an important step toward better understanding of molecular
mechanisms of TRPML channel regulation and physiological function.

In the current study, we characterized, for the first time, the channel function of Drosophila TRPML. When expressed in HEK293 cells, TRPML is present in both lysosomal compartments and plasma membrane. PI(3,5)P2-sensitive inwardly rectifying currents are detectable in both whole-lysosome recordings from vacuoln 1-enlarged vacuoles and inside-out patches excised from plasma membrane. The PI(3,5)P2-elicited current is inhibited by PI(4,5)P2 but not other commonly found phosphoinositides. Using the equivalent Va mutant as that described for mammalian TRPML channels, we demonstrate that TRPML is under bimodal modulation by pH at the extracytosolic side and is permeable to monovalent and divalent cations but blocked by trivalent cations such as Fe3+. B, I-V curves obtained by voltage ramps under conditions shown in A. C and D, similar to A and B, but pH 7.2 Tyrode's solution was used, and 1 mM LaCl3 and 1 mM GdCl3 in the pH 7.2 Tyrode's were applied as shown. E and F, I-V curves obtained by voltage ramps from whole-cell recording of two different TRPMLtransfected cells exposed to pH 4.6 Tyrode's solution without or with 1 mM LaCl3 (E), or GdCl3 (F). G, summary data (means ± S.E.) for % inhibition by La3+ and Gd3+ (both at 1 mM) of whole-cell currents at −100 mV in HEK293 cells that expressed either TRPML or hTRPML. The extracellular solution had pH either at 4.6 or 7.2 as indicated.

In whole lysosome patches, the PI(3,5)P2-elicited current in response to bath application of PI(3,5)P2. This suggests that the plasma membrane TRPML most likely exists in clusters.

The plasma membrane localization of TRPML resembles that of mammalian TRPML2 and TRPML3 (12–14). However, different from these channels, the plasma membrane TRPML channels cannot be activated by exposing cells to a solution that contained no or a low concentration of Na+. Therefore, the TRPML channel does not appear to have the Na+ sensitivity exhibited by the wild type TRPML2 and TRPML3 (12). On the other hand, although it has not been directly determined whether mammalian TRPML1 exhibits sensitivity to extracellular Na+, in whole lysosome patches, the PI(3,5)P2-elicited TRPML1 current was not affected by the luminal Na+ (22) and in whole-cell recordings, extracellular Na+ supported inward currents mediated by the surface expressed TRPML1–4A mutant (23, 36). Therefore, TRPML appears to resemble mammalian TRPML1 for lacking sensitivity to Na+ block. Note that the Na+ sensitivity of TRPML2 and TRPML3 is lost in the Va mutants (13, 15). The Va mutations render all mammalian TRPML channels constitutively active. The equivalent mutation in Drosophila TRPML, A487P, also displays strong constitutive currents in whole-cell recordings.

Also similar to TRPML1 is the Fe2+ permeability of TRPML. Both TRPML1 and TRPML2, have been shown to permeate Fe2+ and TRPML1 was suggested to play a critical role in Fe2+ release from late endosomes and lysosomes, a function severely
impaired in cells from MLIV patients. However, TRPML3 showed little or no Fe\(^{2+}\) permeability (21). We show that TRPML is permeable to Fe\(^{2+}\), with a permeability about 12 times that of Na\(^{+}\). In addition, TRPML also exhibited excellent permeability to other divalent cations, Ca\(^{2+}\) and Mn\(^{2+}\). These properties resemble that of mammalian TRPML1.

In contrast to the permeation to divalent cations, TRPML is impermeable to trivalent iron, Fe\(^{3+}\). Rather, the channel is blocked by Fe\(^{3+}\), as well as lanthanides, La\(^{3+}\) and Gd\(^{3+}\). The block is more pronounced in neutral than acidic pH. This property has not been described for mammalian TRPML channels, although the monovalent conductance of TRPML1 has been shown to be inhibited by Fe\(^{2+}\) (21).

TRPML exhibits equal permeability to Na\(^{+}\), K\(^{+}\), and Cs\(^{+}\), but is impermeable to the large organic monovalent cation, NMDG. The current amplitude displays an order of Na\(^{+}\) > K\(^{+}\) > Cs\(^{+}\) in the absence of divalent cations. The smaller K\(^{+}\) current than Na\(^{+}\) current may be partially explained by the fast inactivation upon switching to the DVF K\(^{+}\) solution (see Fig. 6A).

The pH dependence of TRPML is very interesting. For both the fly TRPML and human TRPML1, we show bell-shaped dependence on extracellular pH, which is equivalent to the pH in lysosomal lumen. However, the pH dependence curve for the human channel is shifted to more acidic range than that for Drosophila TRPML by about 0.6 pH unit. This suggests that optimal activity of TRPML is achieved at less acidic pH than that of mammalian TRPML1. Intriguingly, the lysosomal pH of fly cells is around 5.1 (37), which is more neutral than that of mammalian cells (38, 39). Therefore, both the fly TRPML and mammalian TRPML1 are optimally tuned to the lysosomal environment of the respective cells. Our data suggest that when expressed in human cells, the insect TRPML may be optimally activated in late-endosomes or other organelles that have slightly higher pH than lysosomes. It would be interesting to also know the pH dependence of TRPML2 and TRPML3. At least for TRPML3\(^{Va}\), the constitutive whole-cell current was reported to be smaller at pH 4.6 than at pH 7.4, suggesting an inhibitory effect by strong acid (16). For wild type TRPML3 activated under Na\(^{+}\)-free conditions, acid was also shown to be only inhibitory (14). Therefore, TRPML displays similar pH dependence as mammalian TRPML1, but not TRPML3; however, its pH sensitivity is shifted to less acidic conditions than mammalian TRPML1.

Finally, like all mammalian TRPML channels, TRPML is activated by PI(3,5)P\(_2\) and it displays an inwardly rectifying I-V relationship with very little outward current at positive potentials. These results are consistent with the findings in vivo suggesting that the vesicular trafficking defects observed in the Drosophila trpml\(^{l}\) mutants is remarkably similar to those observed in the Drosophila fab1 mutants, which are unable to synthesize PI(3,5)P\(_2\) (27, 40). The PI(3,5)P\(_2\)-elicited TRPML current is blocked by PI(4,5)P\(_2\), which is consistent with mammalian TRPML1 (23). However, different from TRPML1, TRPML was not blocked by PI(3,4)P\(_2\) or PI(3,4,5)P\(_3\). Thus, although all TRPML channels are regulated by phosphoinositides, the selectivity among different phosphoinositide species is unique for each TRPML. It would be interesting to examine the phosphoinositide sensitivity of TRPML2 and TRPML3.

In conclusion, Drosophila TRPML forms PI(3,5)P\(_2\)-activated inwardly rectifying channels on both endolysosomal and plasma membrane when expressed in HEK293 cells. The channel is specifically inhibited by PI(4,5)P\(_2\), but not other related phosphoinositides. Currents mediated by TRPML are cation non-selective, with excellent permeability to divalent cations, including Ca\(^{2+}\) and Fe\(^{2+}\). The TRPML-mediated current is blocked by trivalent cations, Fe\(^{3+}\), La\(^{3+}\), and Gd\(^{3+}\), and it is strongly potentiated by moderately low pH and inhibited by more acidic pH. Thus, our data suggest that TRPML resembles more closely to mammalian TRPML1 than TRPML2 or TRPML3. Supporting this conclusion, transgenic expression of human TRPML1 coding sequence in the fruit fly partially rescued the pupal lethality phenotype of the trpml\(^{l}\) mutation. The incomplete effect may be explained by the differences between Drosophila TRPML and mammalian TRPML1, such as differential pH dependence, plasma membrane localization, and/or sensitivity to PI(3,4)P\(_2\), and PI(3,4,5)P\(_3\). Collectively, these data support the notion that the Drosophila system serves an excellent model for assessing the functions of TRPML1 in cell physiology and provide molecular basis for understanding some of the differences between mammalian and insect TRPML channels in the regulation of lysosome membrane trafficking, endocytosis and autophagy.

Acknowledgments—We thank Drs. Paul Luzio and Haoxing Xu for cDNA constructs and Alemayehu A. Gerfe for critical data discussion.

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Functional Characterization of Drosophila TRPML

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