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Differential mechanisms of action of the mucolipin synthetic agonist, ML-SA1, on insect TRPML and mammalian TRPML1

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Abstract

Mucolipin synthetic agonist 1 (ML-SA1) was recently identified to activate mammalian TRPML channels and shown to alleviate lipid accumulation in lysosomes of cellular models of lysosome storage diseases, mucopolipidosis type IV (MLIV) and Niemann-Pick's disease type C (NPC). Owing to its potential use in complementing genetic studies in *Drosophila melanogaster* to elucidate the cellular and physiological functions of TRPML channels, we examined the effect of ML-SA1 on *Drosophila* TRPML expressed in HEK293 cells using whole-cell, inside-out, and whole-lysosome electrophysiological recordings. We previously showed that when expressed in HEK293 cells, *Drosophila* TRPML was localized and functional on both plasma membrane and endolysosome. We show here that in both inside-out patches excised from the plasma membrane and whole-lysosome recordings from enlarged endolysosome vacuoles, ML-SA1 failed to activate TRPML unless exogenous phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] was applied. At 1 μM ML-SA1, the sensitivity of TRPML to PI(3,5)P₂ increased approximately by 10-fold and at 10 μM ML-SA1, the deactivation of PI(3,5)P₂-evoked TRPML currents was markedly slowed. On the other hand, constitutive activation of TRPML by a mutation that mimics the varmint-waddler (Va) mutation of mouse TRPML3 rendered the insect channel sensitive to activation by ML-SA1 alone. Moreover, different from the insect TRPML, mouse TRPML1 was readily activated by ML-SA1 independent of PI(3,5)P₂. Thus, our data reveal that while ML-SA1 acts as a true agonist at mouse TRPML1, it behaves as an allosteric activator of the *Drosophila* TRPML, showing dependence on and the ability to stabilize open conformation of the insect channels.

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Keywords

TRPML; lysosome storage disease; TRP channels; allosteric activator; calcium; neurodegeneration

Introduction

Lysosomes are acidic organelles primarily involved in macromolecule degradation, substance recycling and waste extrusion. Genetic mutations that perturb lysosome functions often cause lysosomal storage disease (LSD), such as Niemann-Pick (NP) disease and mucopolipidosis type IV (MLIV) [1, 2]. MLIV is an autosomal recessive LSD, characterized by excessive lysosomal storage of macromolecules, membrane trafficking defects and neurodegeneration [3–6]. The mutated gene in MLIV, MCOLN1, codes for the transient receptor potential mucolipin-1 (TRPML1) protein, which forms an iron (Fe^{2+})- and calcium (Ca^{2+})-permeable channel primarily localized to the late endosomal and lysosomal membranes [7]. TRPML1 mutations are believed to impair Ca^{2+} fluxes and thereby disrupt a number of lysosome functions, including lysosome biogenesis, lysosome trafficking, and substrate digestion, leading to the LSD phenotypes in MLIV patients [8–10].

Intriguingly, TRPML1 expression and function may also be impaired in other forms of LSD. For example, cells from patients with NP disease have similar lysosomal defects as that of TRPML1 mutations, including impairments in autophagosome–lysosome fusion and/or lysosome reformation, defects in lipid trafficking and alterations in Ca^{2+} and Fe^{2+} homeostasis [2,11]. These similarities prompted a functional examination of TRPML1 in cells affected by NP type A and type C (NPA and NPC) mutations and those ablated of the NPC1 gene (NPC1^{−/−} cells). In all cases, the activity of TRPML1 was markedly reduced in the affected as compared to control cells, suggesting a generally reduced TRPML1 function in NP disease [11]. It was further shown that sphingomyelins, which are abnormally accumulated in lysosomes in all NP disease cells, directly inhibited TRPML1 channel activity and moreover, enhancing TRPML1 expression and function by either overexpressing exogenous TRPML1 and/or activating TRPML1 with a small molecular agonist, mucolipin synthetic agonist 1 (ML-SA1) rescued the lysosomal defects in NPC1 mutant cells, including the impaired retrograde transport of lipids from late endosomes and lysosomes to Golgi apparatus, which is commonly found in both TRPML1 and NPC mutations [11]. Therefore, pharmacological intervention of TRPML1, such as the use of TRPML1 agonist, e.g. ML-SA1, may be a good strategy to ameliorate the defective lysosome function in multiple LSD's. However, the mechanism of action of ML-SA1 on TRPML1 remains to be elucidated.

ML-SA1 is a structural analog of SF-51, which was originally identified from a high throughput screen effort for small molecular probes of TRPML3 [12]. ML-SA1 has been shown to activate all three members of the mammalian TRPML subfamily, TRPML1-3 [11] and therefore may be considered as a common agonist of all TRPML channels. Different from vertebrate animals, insects only have one *trpml* gene. However, genetic ablation of the only *trpml* gene in *Drosophila melanogaster* recapitulated many of the cellular defects

found in human MLIV, or TRPML1 mutated, cells [13], implicating a major role for TRPML1 in regulating lysosome function in the mammalian system. This would be consistent with the findings that TRPML2 and TRPML3 have more restricted tissue distributions than TRPML1 [10]. Our recent functional characterization of the *Drosophila* TRPML channel expressed in mammalian cells indeed revealed many similar features between the fly TRPML and mammalian TRPML1, including activation by phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂] but inhibition by phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂], and permeation to Ca²⁺, Fe²⁺, and Mn²⁺, but block by Fe³⁺, as well as bell-shaped extracytosolic pH dependence [14]. On the other hand, there are detectable differences between these channels in subcellular distribution (including the marked presence on the plasma membrane of the fly TRPML), phosphoinositide sensitivity and the optimal pH for channel activity, suggesting that the functional overlap may be partial. Supporting this notion, transgenic expression of human TRPML1 in neurons of the *Drosophila trpml* mutants only partially suppressed the pupal lethality phenotype [14]. Therefore, the fly TRPML could represent a prototypical TRPML channel that encompasses functions served by all mammalian TRPMLs. Since ML-SA1 may be a common activator useful for investigation of physiological functions of TRPML channels in general, we tested whether it also activates the *Drosophila* TRPML and whether the activation occurs through a similar mechanism as to TRPML1.

Here, we show that *Drosophila* TRPML is not activated by ML-SA1 alone, but its activity is strongly potentiated by the compound when the channel is activated by PI(3,5)P₂ or it is constitutively active because of an A → P substitution at A487, which mimics the varitint-waddler (Va) mutation in mouse TRPML3 [14]. These differ from mouse TRPML1, which is readily activated by ML-SA1 in whole-lysosome patches and excised inside-out plasma membrane patches whether or not PI(3,5)P₂ is present, and with the critical PI(3,5)P₂ binding site disrupted by site-directed mutagenesis. Furthermore, we show that structurally distinct agonists identified from the same screen against TRPML3, SF-21, SF-22, and SF-41, have no effect on *Drosophila* TRPML regardless of the stimulating status. Among the three, only SF-22 activated human TRPML1 in whole-lysosome patches.

Materials and Methods

Plasmids and compounds

The constructs for C-terminal GFP-tagged *Drosophila* TRPML (TRPML-EGFP) and TRPML^{Va}-EGFP were created as described previously [14]. The constructs for mouse TRPML1-EGFP, TRPML1-7Q-EGFP, TRPML1-4A-EGFP, TRPML1-4A-7Q-EGFP, TRPML3-EGFP, and human TRPML1-EGFP as described previously [11, 15], were kindly provided by Dr. Haoxing Xu (University of Michigan). TRPML-3Q-EGFP, TRPML-4Q-EGFP, TRPML-3Q^{Va}-EGFP, TRPML-4Q^{Va}-EGFP, and TRPML-7Q^{Va}-EGFP were generated using QuikChange Site-Directed Mutagenesis kit (Stratagene). The final sequences were verified by DNA sequencing.

PI(3,5)P₂ (diC8 form) was purchased from Cayman Chemical Co. ML-SA1 was initially obtained from Vitas-M Laboratory, Ltd (ordered through Molport, Latvia) and subsequently

from Sigma-Aldrich. SF-21, SF-22, and SF-44 were from ENAMINE Ltd (ordered through Molport, Latvia). All other chemicals were from Sigma-Aldrich unless stated otherwise.

Cell culture and transfection

HEK293 culture and transfection were performed as previously described [14]. At 8 hrs post transfection, cells were reseeded on poly-ornithine coated glass coverslips and used within 30 hrs for electrophysiological studies.

Electrophysiology

For whole-cell recordings, recording pipettes were pulled from standard wall borosilicate tubing with filament (Sutter Instrument) to 2–3 M Ω when filled with the pipette solution containing (in mM): 135 Cs-methanesulfonate, 10 CsCl, 1 MgCl₂, 1 EGTA, 4 HEPES (pH 7.2 by CsOH), and placed in the normal Tyrode's bath solution consisted of (in mM): 135 NaCl, 5.6 KCl, 2.6 CaCl₂, 1 MgCl₂, 10 glucose and 10 HEPES (pH 7.4 by NaOH).

For both inside-out and whole-lysosome recordings, the bath solution contained (in mM): 140 K-gluconate, 4 NaCl, 2 MgCl₂, 0.39 CaCl₂, 1 EGTA, 10 HEPES (pH 7.2 by KOH). For inside-out recordings, the pipettes were pulled to 1–2 M Ω when filled with the Tyrode's solution and placed into the bath.

For whole-lysosome recordings, vacuolin-1 (1 μ M, overnight) treated cells were placed in the recording chamber on the stage of an Olympus IX71 inverted fluorescence microscope. A selected EGFP-positive cell that contained enlarged green fluorescence vacuoles was sliced through by a sharp glass pipette mounted to a micromanipulator to release the vacuoles, of which a desired one was captured by the patch pipette filled with the low pH bath solution containing (in mM): 135 NaCl, 5.6 KCl, 2.6 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, and 10 2-morpholinoethanesulfonic acid (pH 4.6 by HCl) and with resistance of ~10 M Ω . After reaching the gigaseal, the membrane was ruptured by a slight suction. Recordings were made with the inside-out mode of the amplifier.

Voltage command and data acquisition were controlled by an EPC10 amplifier using PatchMaster (HEKA electronics Inc., Germany). Typically, currents were elicited by stepping from the holding potential of 0 mV to –100 mV for 10 ms and a voltage ramp from –100 to +100 mV in 200 ms before returning to 0 mV. These were repeated every sec. For voltage steps, 400 ms steps from –80 mV to 80 mV in 10-mV increments and with 3 s intervals were used. Signals were digitized at 5 kHz and filtered at 2 kHz. Drugs 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 μ m from the sample being recorded. The solution exchange was complete within one to two seconds. 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). Summary data are presented as the mean $\pm$ S.E.M. Statistical comparisons were made using Student's *t* test. *P* values of < 0.05 were considered statistically significant.

Result

1. ML-SA1 alone failed to activate *Drosophila* TRPML but potentiated the stimulatory effect of PI(3,5)P₂ on the channel

We previously showed that when expressed in HEK293 cells, *Drosophila* TRPML is present and functional on both plasma membrane and lysosomes when stimulated with PI(3,5)P₂ from the cytoplasmic side [14]. Since ML-SA1 was shown to be a membrane-permeable agonist of all mammalian TRPMLs [11], we tested whether ML-SA1 could evoke similar current as PI(3,5)P₂ in inside-out patches excised from HEK293 cells that expressed *Drosophila* TRPML. To our surprise, no obvious current was seen after bath application (to the cytoplasmic side) of up to 10 μ M ML-SA1, a saturating concentration for activation of mammalian TRPML1 [11], despite the development of robust macroscopic inwardly rectifying current in response to PI(3,5)P₂ (1 μ M) in the same patch (Fig. 1A). On the other hand, when co-applied with PI(3,5)P₂, ML-SA1 (10 μ M) dramatically enhanced the PI(3,5)P₂-evoked current by about 4-fold (3.8 ± 0.4 , $n = 7$), while the current remained inwardly rectifying and reversing near 0 mV (Fig. 1A). The voltage step protocol revealed that the currents evoked by the coapplication of ML-SA1 and PI(3,5)P₂ did not inactivate at all negative potentials (Fig. 1B), just like those elicited by PI(3,5)P₂ alone [14].

To further evaluate the effect of ML-SA1 on TRPML, we recorded TRPML current in enlarged endolysosomes using the whole-lysosome patch clamp method [7, 14, 15]. Cells transfected with TRPML-EGFP were treated with vacuolin-1 (1 μ M, overnight) to enlarge endolysosomal vacuoles. Upon releasing from cells with the help of a sharp glass pipette, the selected vacuole was captured by the recording electrode and used for whole-lysosome recordings. Under these conditions, while bath application of PI(3,5)P₂ (to the cytoplasmic side) evoked TRPML current [14], ML-SA1 (5 μ M) did not (Fig. 1C). However, when ML-SA1 was co-applied with PI(3,5)P₂ (0.1 μ M), it enhanced the phospholipid-evoked current (Fig. 1D). This effect of ML-SA1 is similar to that seen in the inside-out patches, suggesting that ML-SA1 is not a true agonist, but rather an allosteric modulator that potentiates the stimulating effect of PI(3,5)P₂ on *Drosophila* TRPML. The fact that ML-SA1 failed to evoke TRPML current not only in inside-out plasma membrane patches after extended perfusion but also from whole-lysosome patches, both of which should have minimal levels of PI(4,5)P₂, indicates that the failure of ML-SA1 to activate TRPML was not caused by an inhibition from endogenous PI(4,5)P₂.

To gain more information about the potentiating effect of ML-SA1 on PI(3,5)P₂-induced TRPML activation, we compared the concentration dependence on PI(3,5)P₂ of TRPML currents in inside-out plasma membrane patches in the absence and presence of ML-SA1. Previously, we have obtained an EC₅₀ value of 390 nM for PI(3,5)P₂ activation of TRPML in the absence of ML-SA1 and the current was not elicited at concentrations lower than 30 nM under this configuration [14]. With the coapplication of 20 μ M ML-SA1, obvious TRPML currents were elicited by as low as 1 nM PI(3,5)P₂ and the current reached saturation at >10 nM of the phospholipid, implying a full activation of *Drosophila* TRPML by 10 nM PI(3,5)P₂ in the presence of 20 μ M ML-SA1 (Fig. 2A). Reducing the ML-SA1 concentration to 1 μ M diminished the stimulating effect of 1 nM PI(3,5)P₂, but still allowed

10 nM PI(3,5)P₂ to elicit a small TRPML current (Fig. 2B, C). The EC₅₀ for PI(3,5)P₂ in the presence of 1 μM ML-SA1 was determined to be 37.5 ± 4.7 nM (n = 5) (Fig. 2C), which represents a ~10-fold increase in the affinity of PI(3,5)P₂ to stimulate TRPML as compared to in the absence of ML-SA1 reported previously [14].

2. ML-SA1 slowed down the dissociation of PI(3,5)P₂ from *Drosophila* TRPML

Both activation and deactivation of *Drosophila* TRPML by PI(3,5)P₂ were relatively fast in the inside-out patches. The presence of ML-SA1 (10 μM) slowed down the deactivation upon washout of PI(3,5)P₂ (1 μM) (Fig. 3A). In these experiments, after eliciting TRPML current with 10 μM ML-SA1 plus 1 μM PI(3,5)P₂, the patch was exposed to either 10 μM ML-SA1 or 1 μM PI(3,5)P₂ alone. The deactivation kinetics was assessed by fitting the time course of current decrease at -100 mV with the Boltzmann equation to yield the time constant (tau) of deactivation (Fig. 3A, B). While excluding ML-SA1 from the perfusion solution resulted in a rapid decrease of TRPML current with the time constant of 1.07 ± 0.18 s (n = 6), the removal of PI(3,5)P₂ from the co-stimulating solution caused a much slower decrease of the current, with the time constant of 16.4 ± 3.1 s (n = 6). This value is significantly higher than that obtained in the absence of ML-SA1 for the same concentration of PI(3,5)P₂ (tau = 1.74 ± 0.41 s, n = 5) or upon washout of PI(3,5)P₂ one minute after ML-SA1 had been removed (tau = 3.6 ± 1.4 s, n = 5). In addition to the slower deactivation upon removal of PI(3,5)P₂ in the continued presence of ML-SA1, the current did not return to the baseline, with significant current left after 2 min exposure to ML-SA1 alone (Fig. 3A). This suggests that either PI(3,5)P₂ could not be completely washed out (dissociated) from TRPML in the presence of ML-SA1 or the drug might be better at binding to the open than to the closed conformation of the TRPML channel. The former interpretation would be consistent with the idea that ML-SA1 slowed down the dissociation of PI(3,5)P₂ from the channel, in line with the observed increase in the deactivation time constant. As a comparison, the removal of ML-SA1 from the co-stimulating solution returned the current amplitude to slightly above that induced by PI(3,5)P₂ alone, suggesting that the washout of ML-SA1 was quite complete under our experimental conditions. Together, the above data demonstrated that ML-SA1 potentiated PI(3,5)P₂-induced TRPML channel activation either by slowing down the dissociation of the phospholipid or by binding to and stabilizing the open channel conformation opened by PI(3,5)P₂.

3. ML-SA1 enhanced currents of *Drosophila* TRPML^{Va} mutant

In order to examine whether PI(3,5)P₂ was necessary for the potentiating effect of ML-SA1, we used the TRPML^{Va} mutant (A487P), which mimics the varitint-waddler (Va) mutant of mouse TRPML3, exhibiting constitutive whole-cell currents [7, 14]. Expression of *Drosophila* TRPML^{Va}-EGFP in HEK293 cells resulted in a marked constitutive inwardly rectifying whole-cell current (Fig. 4A), which reversed at near 0 mV and was characteristic of TRPML currents. Interestingly, application of ML-SA1 (1–10 μM) from the extracellular side significantly enhanced the constitutive basal current in a concentration-dependent manner (Fig. 4A, B). These results suggested that ML-SA1 might enhance *Drosophila* TRPML function through interaction with the open channel conformation in the absence of PI(3,5)P₂. However, it could not be excluded that a trace amount of PI(3,5)P₂ present in the plasma membrane might have contributed to TRPML^{Va} potentiation by ML-SA1, given that

as low as 1 nM PI(3,5)P₂ was able to evoke TRPML current in the presence of 20 μM ML-SA1 in inside-out patches (Fig. 2A).

To rule out the possibility of contribution by a trace amount of PI(3,5)P₂ in the plasma membrane, we substituted the positively charged amino acids at the N-terminus of *Drosophila* TRPML with glutamines. A previous study on mammalian TRPML1 had identified seven N-terminal positively charged residues as the critical sites for PI(3,5)P₂ sensing [15]. However, these are not entirely conserved with respect to the last lysine and two arginines based on the sequence alignment (Fig. 4C). Therefore, we mutated R96, R97, K98, K109, K113, R114 and K115 of TRPML^{Va} to glutamines (TRPML-7Q^{Va}). Strikingly, no basal TRPML-like current was detected by whole-cell recordings (n = 5) and ML-SA1 (10 μM) did not evoke any current (whole-cell, n=3; inside-out, n=10), indicating that TRPML-7Q^{Va} may be non-functional. To overcome this problem, we made two other mutants, TRPML-3Q^{Va}, and TRPML-4Q^{Va}, with only the first three (R96Q, R97Q and K98Q) and the last four (K109Q, K113Q, R114Q and K115Q) of the seven positively charged residues substituted by glutamines, respectively. Both mutants showed constitutive basal currents, albeit being much smaller than that of TRPML^{Va} (Fig. 4D–F). Importantly, ML-SA1 (10 μM) significantly enhanced the basal currents of TRPML-3Q^{Va} and -4Q^{Va}, even to a large extent than TRPML^{Va} (Fig. 4G). To test the sensitivity of 3Q and 4Q mutations to PI(3,5)P₂, we measured the response to bath applications of PI(3,5)P₂ (1 μM) and PI(3,5)P₂ + ML-SA1 (20 μM) of inside-out patches excised from HEK293 cells that expressed TRPML-3Q-EGFP or TRPML-4Q-EGFP. As shown in Fig. 4I, J, either mutant responded to PI(3,5)P₂ or PI(3,5)P₂ + ML-SA1 while the WT TRPML-EGFP, which was tested in parallel, responded robustly to the same treatments (Fig. 4H). Remarkably, without the constitutive activity caused by the Va mutation, the TRPML-3Q and TRPML-4Q mutants not only failed to respond to PI(3,5)P₂, but also was unresponsive to ML-SA1. The linear current-voltage (I–V) relationships observed for these patches, which likely represent leak currents, suggest also a lack of constitutive activity. These results argue against the idea that a trace amount of PI(3,5)P₂ in the plasma membrane could contribute to ML-SA1 potentiation of TRPML^{Va} activity, but suggest that ML-SA1 exerts its potentiating effect via open channels.

4. ML-SA1 activated mammalian TRPML1 independent of activity

The findings above in *Drosophila* TRPML prompted us to ask whether ML-SA1 is a true agonist of mammalian TRPML1. Consistent with the results by Shen et al. [11], whole-lysosome recordings from enlarged vacuoles released from HEK293 cells that expressed mouse TRPML1-EGFP (mTRPML1) showed that application of either PI(3,5)P₂ (1 μM) or ML-SA1 (0.3 μM) to the cytoplasmic side of the endolysosome induced inwardly rectifying current (Fig. 5A). Co-application of ML-SA1 and PI(3,5)P₂ showed an additive effect compared to the application of individual compound alone (Fig. 5C). Therefore, ML-SA1 activated mTRPML1 in the absence of exogenously applied PI(3,5)P₂. However, since the mammalian TRPML1 has been shown to have a higher affinity to PI(3,5)P₂ than *Drosophila* TRPML (EC₅₀ ~ 48 nM [15]), the activation of mTRPML1 by ML-SA1 could be facilitated by the residual endogenous PI(3,5)P₂ in the enlarged endolysosome vacuoles. To rule out this possibility, we recorded ML-SA1-evoked currents in endolysosomes isolated from

HEK293 cells that expressed mTRPML1-7Q-EGFP, which had all seven critical N-terminal residues for PI(3,5)P₂ binding mutated to glutamines and displayed greatly reduced response to PI(3,5)P₂ [15]. Notably, although this mutant exhibited no detectable basal current and was unresponsive to PI(3,5)P₂ (1 μM) or a low concentration of ML-SA1 (0.3 μM), it responded robustly to 10 μM ML-SA1 in whole-lysosome patches, with an I–V relationship typical of TRPML channels (Fig. 5B, D). Therefore, different from the insect TRPML, the activation of mammalian TRPML1 channels by ML-SA1 does not require costimulation by PI(3,5)P₂ or constitutive channel activation.

Since the lysosome is enriched of PI(3,5)P₂, the residual endogenous PI(3,5)P₂ on the patched lysosomes could influence the effect of ML-SA1. To exclude this possibility, we used the mutant mTRPML1-4A (L15, L16, L577, L578 changed to alanines), which does not bear the dileucine motifs in the N- and C-termini and was thus highly expressed on the plasma membrane [16]. As previously reported [11], bath application of ML-SA1 evoked robust inwardly rectifying whole-cell currents in HEK293 cells that expressed mTRPML1-4A (data not shown). In inside-out patches excised from these cells, bath application of 10 μM ML-SA1 alone induced inwardly rectifying currents (Fig. 6A), with a similar I–V relationship as that recorded from whole-lysosomes for WT mTRPML1 (compare with Fig. 5A). Strikingly, although PI(3,5)P₂ (1 μM) did not evoke any appreciable current under these conditions, it strongly potentiated that activated by ML-SA1 in the inside-out patches (Fig. 6A). More importantly, ML-SA1 also activated mTRPML1-4A-7Q in the inside-out patches and this effect was not affected by the co-application of PI(3,5)P₂ (Fig. 6B), consistent with 7Q being insensitive to PI(3,5)P₂. These results further support the conclusion that ML-SA1 can stimulate mammalian TRPML1 in an activity independent manner.

5. Structurally distinct mammalian TRPML agonists, SF-21, SF-22, and SF-41, had no effect on *Drosophila* TRPML

In addition to ML-SA1, SF-22 and its improved analog, MK6-83, have been shown to activate human TRPML1 and rescue the lysosomal trafficking defects and zinc accumulation in the fibroblasts of MLIV patients [17]. To learn if SF-22 or another mammalian TRPML agonist could activate the insect TRPML, we tested SF-21, SF-22, and SF-41 on *Drosophila* TRPML expressed in HEK293 cells using inside-out patches. All three drugs were originally identified from the same high throughput screen against TRPML3, which also yielded the lead compound for ML-SA1 [12]. We have confirmed that they all activated mouse TRPML3 expressed in HEK293 cells by whole-cell recordings (data not shown). However, at 10 μM, none of them activated *Drosophila* TRPML in the inside-out patches, despite the robust response to PI(3,5)P₂ (Fig. 7A). In addition, co-application of SF-21, SF-22, or SF-41 with PI(3,5)P₂ did not alter the response of TRPML to PI(3,5)P₂, while ML-SA1 increased the response by approximately 1.5 fold (Fig. 7B, C). As a control, we showed that SF-22 (10 μM) activated human TRPML1 expressed in HEK293 cells in whole-lysosome patches, although the potency was lower than ML-SA1 at the same concentration (Fig. 7Di). On the other hand, SF-21 and SF-41 (both at 10 μM) did not activate human TRPML1 (Fig. 7Dii). These data suggest that the newly identified TRPML

probes are species and isoform specific and the ability of ML-SA1 to potentiate the fly TRPML channel is a unique property of this compound.

Discussion

Recent studies have revealed the importance of TRPML channels in endolysosomal functions, including substance degradation, ion homeostasis, and vesicle trafficking. Not only genetic mutations in TRPML1 cause LSD and neurodegeneration [2], but also other LSD's bear impaired TRPML function [11], implicating a pivotal role of TRPML channels in maintaining the normal cellular function through their actions on the endolysosome system. The successful remission of the lysosomal defects of NPC1 mutant cells by the small molecular TRPML agonist, ML-SA1, testifies the utility of drug intervention of TRPML function in the treatment of LSD [11]. However, the mechanisms of action of ML-SA1 on TRPML channels warrant careful evaluation.

We show here that ML-SA1 acted differently on the *Drosophila* TRPML channel than on mouse TRPML1. While ML-SA1 activated wild type TRPML1 readily in whole-lysosome recordings and the cell surface-targeted TRPML1 mutant, TRPML1-4A, in inside-out patches excised from the plasma membrane, it did not activate the insect TRPML under neither recording configuration, despite the use of the same host system for heterologous expression. However, ML-SA1 strongly potentiated TRPML channel function in whole-lysosome recordings and inside-out patches when the channel was stimulated by its endogenous agonist, PI(3,5)P₂. In the inside-out patches, the presence of ML-SA1 strongly increased the sensitivity of TRPML channel to PI(3,5)P₂, causing a more than 10-fold shift of the apparent affinity with as low as 1 μM ML-SA1, suggesting that although ML-SA1 is not a true agonist of TRPML, it can act as an allosteric modulator to enhance PI(3,5)P₂-evoked TRPML channel activity. However, the potentiating effect of ML-SA1 is not absolutely dependent on PI(3,5)P₂. With the Va mutation that renders the channel constitutively active, ML-SA1 enhanced TRPML whole-cell currents in the absence of any exogenously applied PI(3,5)P₂. Therefore, the effect of ML-SA1 on TRPML1 appears to be state-dependent, with the Va mutation mimicking, perhaps, the PI(3,5)P₂-bound conformation that allows ML-SA1 to bind and activate the channel.

Intriguingly, the endogenous PI(3,5)P₂, which was thought to be present at low levels on the enlarged endolysosome vacuoles, was not sufficient to support TRPML activation by ML-SA1. It is possible that the endogenous PI(3,5)P₂ levels were simply too low to synergize with ML-SA1 in the enlarged vacuoles; however, they must be quite lower than 1 nM equivalent of the exogenously applied diC8 PI(3,5)P₂, which led to obvious current in the presence of 20 μM ML-SA1 even in inside-out plasma membrane patches, where inhibition by endogenous PI(4,5)P₂ should negatively impact the current development [14]. This would be consistent with the idea that PI(3,5)P₂ in quiescent cells is kept at extremely low levels by the concerted actions of phosphoinositide kinase, PIKfyve, and the Sac1 domain-containing PI(3,5)P₂ 5-phosphatase, Sac3 [18, 19]. In the complex formed by PIKfyve, ArPIKfyve, and Sac3, the newly synthesized PI(3,5)P₂ may be quickly reverted to its precursor, PI(3)P [19, 20], thus maintaining the low levels of PI(3,5)P₂. Furthermore, reducing the PI(3,5)P₂ level is known to induce vacuolation [21–23]. Although the

mechanism of action of vacuolin-1 remains unclear, it is possible that the enlarged endolysosome vacuoles naturally contained much less endogenous PI(3,5)P₂ than regular endolysosomes. Thus, our results revealed very little, if any, contribution of endogenous PI(3,5)P₂ in the activation of *Drosophila* TRPML or mouse TRPML1 in electrophysiological recordings from either whole-lysosomes or inside-out plasma membrane patches. The lack of PI(3,5)P₂ in plasma membrane patches is expected since the phospholipid is mainly found in endolysosomal membranes [24, 25].

The absolute dependence on PI(3,5)P₂ was also supported by the lack of effect of a very high concentration of ML-SA1 (20 μM) on TRPML-3Q and TRPML-4Q in the presence of 1 μM PI(3,5)P₂. These mutants should have reduced PI(3,5)P₂ sensitivity because of the loss of N-terminal positively charged residues [15]. On the other hand, the introduction of Va mutation in these mutants, TRPML-3Q^{Va} and TRPML-4Q^{Va}, rendered them constitutively active and sensitive to stimulation by ML-SA1 even in the whole-cell configuration, indicating that not only can the 3Q and 4Q mutants be functional, but also the drug can act on the active TRPML channels independent of PI(3,5)P₂. Our data, thus, suggest that ML-SA1 only acts on open TRPML channels, which are either activated by PI(3,5)P₂ or rendered constitutively active by a mutation. Most likely, the drug acts by stabilizing the open conformation. This explains the slower deactivation kinetics upon washout of PI(3,5)P₂ in the presence of ML-SA1.

Contrary to the insect channel, however, ML-SA1 activated mouse TRPML1 in the absence of PI(3,5)P₂ or any detectable constitutive activity. This is most evident by the effect of ML-SA1 on TRPML1-7Q, which has a greatly compromised sensitivity to PI(3,5)P₂ [15] and no detectable basal current in whole-lysosome recordings. For both endolysosome localized TRPML1-7Q and plasma membrane-targeted TRPML1-4A-7Q, ML-SA1 evoked typical TRPML1-like currents. As expected from its lack of sensitivity to the phosphoinositide, co-application of 1 μM PI(3,5)P₂ did not alter the current amplitude of TRPML1-4A-7Q evoked by ML-SA1. However, for TRPML1-4A, the presence of ML-SA1 enhanced the ability of PI(3,5)P₂ to activate the channel in inside-out plasma membrane patches. For the wild type channel, PI(3,5)P₂ and ML-SA1 also showed an additive effect in whole-lysosome recordings. Therefore, ML-SA1 appears to be a true agonist of mammalian TRPML1, which evokes channel activity independent of PI(3,5)P₂ and the activation state of the channel. This difference in the mechanisms of action between the fly TRPML and mammalian TRPML1 probably lies in the subtle structural variations between the two channels in the closed states. Perhaps, ML-SA1 is able to bind to the closed state of TRPML1 and induce its activation but not able to do so to TRPML unless an open conformation is established. However, once bound, the mechanism of action on the two channels may be the same, i.e. the stabilization of the open conformation. Therefore, although the current study focuses on the insect channel, it may also reveal the mechanism of action of ML-SA1 on the mammalian counterparts. It is rather intriguing that at rest mammalian TRPML1 and *Drosophila* TRPML may exist in different conformations, with only the latter being unrecognizable by ML-SA1. However, once activated, the two channel types may adapt a similar conformation, in which the drug can readily bind. This differs from the more common scenario in which the affinity difference due to variations in the residues involved

in ligand binding defines the species and isoform preference of a drug. Consistent with such a mechanism, we found SF-22 to exhibit no activity on *Drosophila* TRPML, but stimulate human TRPML1 as shown by others [17]. In addition, SF-21 and SF-41 activated TRPML3 but not TRPML1 or the insect TRPML. Therefore, ML-SA1 is rather unique in its ability to affect multiple distantly related TRPML channels. This suggests that ML-SA1 must bind to a relatively conserved region, likely the pore area of the channel. However, it cannot be ruled out at this point that ML-SA1 might also act indirectly at the channels by binding to an endogenous protein that regulates all TRPMLs. Further studies are needed to examine these possibilities.

Notably, the TRPML currents in inside-out patches were quite large. This was in part due to the large membrane area captured by the 1–2 M Ω pipette tips as well as the uneven distribution of the channel on the plasma membrane. Previously, by using the tip sizes of 2–3 M Ω , we could also record the similar size currents for *Drosophila* TRPML in inside-out patches. However, the success rate was only ~50% [14], suggesting that the channels are clustered. The larger size tips have improved the success rate and increased the mean current amplitude. The other reason for the large “disproportional” currents in inside-out configuration was due to the washout of inhibitory factors, which could be present in the whole-cell configuration and suppress channel activity. One such factor is PI(4,5)P₂, which we have shown to inhibit TRPML and be gradually washed out in the inside-out recordings [14]. Disproportionally large inside-out currents have also been reported for other heterologously expressed channels, e. g. TRPM4 [26].

In summary, we show that the newly described small molecular TRPML agonist, ML-SA1, acts differently on *Drosophila* TRPML and mouse TRPML1. While it behaved as a true agonist of TRPML1, it worked only as an allosteric activator of TRPML, for which the activation by PI(3,5)P₂ or constitutive activity was a prerequisite for the stimulatory action of ML-SA1. Since targeting TRPML channels represents a promising therapeutic strategy to treat LSD [11], detailed understanding on the mechanism of action of TRPML drugs will not only help advance investigations of the physiological and pathological functions of these channels but also facilitate the design of better pharmaceutical agents useful to combat these diseases.

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Abbreviations used

I–V	current-voltage
ML-SA1	Mucolipin synthetic agonist 1
MLIV	mucolipidosis type IV

LSD	lysosomal storage disease
NP	Niemann-Pick's
NPA	NP disease type A
NPC	NP disease type C
PI(3,5)P₂	phosphatidylinositol 3,5-bisphosphate
PI(4,5)P₂	phosphatidylinositol 4,5-bisphosphate
TRPML1	transient receptor potential mucolipin-1 (TRPML1)
Va	varitint-waddler mutant

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Highlights

- TRPML channels are important for lysosome trafficking and function
- Studying *Drosophila* TRPML has revealed important information on TRPML function
- ML-SA1 is a new agonist of mammalian TRPML channels
- We found ML-SA1 only enhanced activity of open but not closed *Drosophila* TRPML
- We found ML-SA to be a true agonist of mammalian TRPML1

Figure 1. ML-SA1 failed to activate *Drosophila* TRPML but potentiated the TRPML currents induced by PI(3,5)P₂ in inside-out and whole-lysosome patches

Drosophila TRPML was expressed in HEK293 cells by transient transfection and currents were recorded from either inside-out patches excised from the plasma membrane (**A & B**) or enlarged endolysosome vacuoles isolated from cells treated with vacuolin-1 (1 μ M, overnight) (**C & D**). **A.** Responses of an inside-out (I/O) patch to bath (cytoplasmic side) applications of PI(3,5)P₂ (1 μ M) and ML-SA1 (1, 3, 10 μ M) individually and coapplication of PI(3,5)P₂ and ML-SA1 (10 μ M). The patch was held at 0 mV; voltage ramps from -100 mV to +100 mV for 200 ms at 1 sec intervals were used to elicit currents while the drugs were applied as indicated. *Left panel shows* time courses of currents at -100 mV and +100 mV. Dashed line indicates zero current. *Right panel shows* current-voltage (I-V) curves obtained by the voltage ramp during the application of 10 μ M ML-SA1 (*dashed black line*), 1 μ M PI(3,5)P₂ (*solid grey line*) and 10 μ M ML-SA1 plus 1 μ M PI(3,5)P₂ (*solid black line*). **B.** Currents elicited in an I/O patch by voltage steps (-80 mV to +80 mV at 10 mV increments from the holding of 0 mV and with a step duration of 400 ms) before (*no drug*, upper traces) and during application of 10 μ M ML-SA1 and 1 μ M PI(3,5)P₂ (*lower traces*). **C.** Responses of a whole-lysosome (W/L) patch to bath (cytoplasmic side) application of ML-SA1 (5 μ M) alone and coapplication of PI(3,5)P₂ (0.1 μ M) and ML-SA1 (5 μ M) as indicated. The same voltage ramp protocol was used as in (**A**). *Left panel shows* time courses of currents at -100 mV and +100 mV. Dashed line indicates zero current. *Right panel shows* I-V curves obtained by the voltage ramp before drug application (*no drug*, *solid grey line*) and during the application of 5 μ M ML-SA1 (*dashed black line*) and 5 μ M ML-SA1 plus 0.1 μ M PI(3,5)P₂ (*solid black line*). **D.** Summary of current amplitudes at -100 mV induced by ML-SA1, PI(3,5)P₂, and ML-SA1 plus PI(3,5)P₂ in whole-lysosome patches recorded as in (**C**). Data are means $\pm$ SEM for n = 3 vacuoles. *, p < 0.01 by paired *t* test.

Figure 2. ML-SA1 enhanced the sensitivity of *Drosophila* TRPML1 to PI(3,5)P₂

Drosophila TRPML was expressed in HEK293 cells by transient transfection and currents were recorded from inside-out (I/O) patches excised from the plasma membrane. The same voltage ramp protocol as in Fig. 1A was used. **A.** Responses of an I/O patch to bath (cytoplasmic side) applications of varying concentrations of PI(3,5)P₂ in the continued presence of 20 μM ML-SA1 as indicated. *Left panel shows* time courses of currents at -100 mV and +100 mV. Dashed line indicates zero current. *Right panel shows* I-V curves obtained by the voltage ramp during the application of 20 μM ML-SA1 alone (indicated as 0, solid grey line) and coapplication of 20 μM ML-SA1 with 1 nM (dashed black line), 10 nM (dashed grey line), and 100 nM (dashed black line) PI(3,5)P₂ as pointed by arrows. Note the nearly complete overlap between currents for 10 and 100 nM PI(3,5)P₂. **B.** Responses of an I/O patch to bath applications of varying concentrations of PI(3,5)P₂ in the continued presence of 1 μM ML-SA1 as indicated. *Left panel shows* time courses of currents at -100 mV and +100 mV. Dashed line indicates zero current. *Right panel shows* I-V curves

obtained by the voltage ramp during the application of 1 μ M ML-SA1 alone (indicated as 0) and coapplication of 1 μ M ML-SA1 with varying concentrations of PI(3,5)P₂ as indicated.

C. Concentration dependence of PI(3,5)P₂-evoked currents in the presence of 1 μ M ML-SA1. Current amplitudes at -100 mV were normalized to that elicited by 300 nM PI(3,5)P₂ and 1 μ M ML-SA1. Data points (means $\pm$ SEM, n = 5) were fitted with the Hill equation, which yielded an EC₅₀ of 37.5 ± 4.7 nM.

Figure 3. ML-SA1 slowed down the deactivation of PI(3,5)P₂-evoked TRPML currents
Drosophila TRPML was expressed in HEK293 cells by transient transfection and currents were recorded from inside-out (I/O) patches excised from the plasma membrane. The same voltage ramp protocol as in Fig. 1A was used. **A.** Time courses of currents at -100 mV and $+100$ mV in an I/O patch during application and washout of PI(3,5)P₂ ($1 \mu\text{M}$) and ML-SA1 ($10 \mu\text{M}$) as indicated. Dashed line indicates zero current. **B.** Summary (means $\pm$ SEM) of deactivation time constants (τ_{deact}) during the washout of ML-SA1 and the washout PI(3,5)P₂ (PIP₂) in the absence or presence of ML-SA1. Current decays at -100 mV were fitted by the Boltzmann equation: $y = (a_1 - a_2) / [1 + e^{-(x - x_0)/\tau}] + a_2$; where: a_1 and a_2 represent the initial and final currents (y), respectively, τ is the time constant for the decay, and $x - x_0$ is the time elapsed. n , the number of patches; **, $p < 0.05$ by unpaired t test.

Figure 4. ML-SA1 enhanced the activity of constitutively active *Drosophila* TRPML mutants independent of PI(3,5)P₂

A. *Drosophila* TRPML^{Va} mutant was expressed in HEK293 cells by transient transfection and whole-cell (W/C) currents were recorded using voltage ramps from -100 mV to +100 mV for 200 ms at 1 sec intervals with the holding potential of 0 mV. Shown are I-V curves obtained by the voltage ramp from a representative cell before (indicated as 0) and during applications of 1, 3, and 10 μM ML-SA1 to the bath (extracellular side). **B.** Summary (means ± SEM) of current amplitudes at -100 mV normalized to that in the absence of ML-SA1 for n = 4 cells as exemplified in (A). **, p < 0.05 by paired t test. **C.** Alignment of N-terminal regions of *Drosophila* (Dm) TRPML and mouse (Mm) TRPML1 and TRPML2. The shaded residues in MmTRPML1 indicate the positively charged amino acids mutated in TRPML1-7Q to render it insensitive to PI(3,5)P₂ [15]. Residues mutated to glutamines (Q) in DmTRPML are indicated by the solid horizontal lines on top of the alignment. The mutation of the first three R/K to Q yielded TRPML-3Q while that of the latter four R/K made TRPML-4Q; the combination of 3Q and 4Q generated TRPML-7Q. Numbers indicate positions in the full-length sequences. **D & E.** The expression of TRPML-3Q^{Va} (D) and TRPML-4Q^{Va} (E) in HEK293 cells led to constitutive currents which were further increased by the bath application of ML-SA1 (10 μM) in whole-cell recordings performed as in (A). Shown are I-V curves obtained by the voltage ramp from representative cells before (basal) and during application of ML-SA1. **F.** Summary of basal whole-cell currents at -100 mV in cells that expressed TRPML^{Va}, TRPML-3Q^{Va}, and TRPML-4Q^{Va}. Data are means ± SEM for the numbers of cells indicated. **G.** Summary (means ± SEM) of fold increase induced by ML-SA1 (10 μM) of whole-cell currents at -100 mV in cells that expressed TRPML^{Va}, TRPML-3Q^{Va}, and TRPML-4Q^{Va}. **H-J.** TRPML-3Q and TRPML-4Q had compromised response to PI(3,5)P₂. Inside-out (I/O) patches excised from HEK293 cells transiently expressing *Drosophila* TRPML (H), TRPML-3Q (I), or TRPML-4Q (J) were examined

under unstimulated conditions and when stimulated with 1 μM PI(3,5)P₂ or 1 μM PI(3,5)P₂ plus 20 μM ML-SA1 using the same voltage ramp protocol as in Fig. 1A. Shown are I–V curves obtained by the voltage ramp from representative patches before (*no drug*) and during application of PI(3,5)P₂ or PI(3,5)P₂ plus ML-SA1. Note that the 3Q and 4Q mutants did not develop any current even with the coapplication of PI(3,5)P₂ and ML-SA1.

Figure 5. ML-SA1 activated mouse TRPML1 in whole-lysosome patches independent of PI(3,5)P₂

Mouse TRPML1 (**A** & **C**) or TRPML1-7Q (**B** & **D**) was expressed in HEK293 cells by transient transfection and currents were recorded from enlarged endolysosome vacuoles isolated from cells treated with vacuolin-1 (1 μM , overnight) using the same voltage ramp protocol as in Fig. 1C. PI(3,5)P₂ and ML-SA1 were applied through bath (cytoplasmic side) perfusion. **A** & **B**. Representative traces of time courses of whole-lysosome (W/L) currents at -100 mV and +100 mV, with the durations and concentrations of PI(3,5)P₂ and ML-SA1 applications indicated on top (*Left panels*), and I-V curves obtained by the voltage ramp at the time points labeled (*Right panels*) for cells that expressed mTRPML1 (**A**) and mTRPML1-7Q (**B**). Dashed lines indicate zero current. **C**. Summary of current amplitudes at -100 mV induced by 1 μM PI(3,5)P₂, 0.3 μM ML-SA1, and 1 μM PI(3,5)P₂ plus 0.3 μM ML-SA1, normalized to the maximal currents evoked by PI(3,5)P₂ and ML-SA1 together. Data are means $\pm$ SEM for n = 3 vacuoles from cells that expressed mTRPML1. **D**. Summary of current amplitudes at -100 mV induced by 1 μM PI(3,5)P₂, 0.3 μM ML-SA1,

and 10 μ M ML-SA1 in vacuoles from cells that expressed mTRPM1-7Q. Data are means $\pm$ SEM for the indicated numbers of vacuoles.

Figure 6. ML-SA1 activated the plasma membrane targeted mouse TRPML1-4A and mTRPML1-4A-7Q mutants in inside-out patches independent of PI(3,5)P₂

A & B. Mouse TRPML1-4A (**A**) or TRPML1-4A-7Q (**B**) was expressed in HEK293 cells by transient transfection and currents were recorded from inside-out (I/O) patches excised from the plasma membrane using the same voltage ramp protocol as in Fig. 1A. PI(3,5)P₂ (1 μ M) and ML-SA1 (10 μ M) were applied through bath (cytoplasmic side) perfusion. Shown are representative traces of time courses for currents at -100 mV and +100 mV, with the durations of PI(3,5)P₂ and ML-SA1 applications indicated on top (*Left panels*), and I-V curves obtained by the voltage ramp at the time points labeled (*Right panels*). Dashed lines indicate zero current. **C.** Summary of current amplitudes at -100 mV induced by 1 μ M PI(3,5)P₂ and 10 μ M ML-SA1 in I/O patches excised from cells that expressed mTRPML1-4A (4A, *left group*) and mTRPML1-4A-7Q (4A-7Q, *right group*). Data are means $\pm$ SEM for the indicated numbers of patches. **D.** Ratio of current amplitude at -100 mV induced by 10 μ M ML-SA1 (SA1) plus 1 μ M PI(3,5)P₂ (PIP₂) to that induced by 10 μ M ML-SA1 alone for I/O patches excised from cells that expressed mTRPML1-4A (4A) and mTRPML1-4A-7Q (4A-7Q). Shown are means $\pm$ SEM for n = 3 patches. A ratio of 1 indicates no facilitation by PI(3,5)P₂. *, p < 0.05 different from the value 1, by one-sample t test.

Figure 7. SF-21, SF-22, and SF-41 had no effect on *Drosophila* TRPML

Drosophila TRPML (A–C) or human TRPML1 (D) was expressed in HEK293 cells by transient transfection and currents were recorded from inside-out (I/O) patches excised from the plasma membrane (A–C) or enlarged endolysosome vacuoles isolated from cells treated with vacuolin-1 (D). The same voltage ramp protocol as in Fig. 1A was used. **A.** Responses of an I/O patch to bath (cytoplasmic side) applications of PI(3,5)P₂ (1 μ M) and SF compound individually (all at 10 μ M). *Left panel shows* time courses of currents at –100 mV and +100 mV. Dashed line indicates zero current. *Right panel shows* I–V curves obtained by the voltage ramp during the application of the indicated drugs. Representative of six I/O patches with similar results. **B.** I–V curves for an I/O patch exposed to 1 μ M PI(3,5)P₂ alone and the same concentration of PI(3,5)P₂ together with 10 μ M SF-21, SF-22, SF-41, or ML-SA1. Note, only ML-SA1 increased the PI(3,5)P₂-evoked current at negative potentials. **C.** Summary of current amplitudes at –100 mV normalized to that induced by 1 μ M PI(3,5)P₂ alone (*dashed line*). **D.** I–V curves for whole-lysosome patches expressing human (h)TRPML1 exposed to 10 μ M SF-22 (Di), SF-21, or SF-41 (Dii). ML-SA1 (10 μ M) was used as a control. Inset in Di shows summary of current amplitudes at –100 mV normalized to that induced by ML-SA1 (all at 10 μ M).