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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, mucolipidosis type IV (MLIV) and Niemann–Pick's disease type C (NPC). Owning to its potential use in complimenting 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 [$PI(3,5)P_2$] was applied. At 1 μM ML-SA1, the sensitivity of TRPML to $PI(3,5)P_2$ increased approximately by 10-fold and at 10 μM ML-SA1, the deactivation of $PI(3,5)P_2$-evoked TRPML currents was markedly slowed. On the other hand, constitutive activation of TRPML by a mutation that mimics the varitint-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 $PI(3,5)P_2$. 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.

1. 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 mucolipidosis 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+} homoeostasis [2,11]. These similarities

Abbreviations: *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; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; TRPML1, transient receptor potential mucolipin-1 (TRPML1); Va, varitint-waddler mutant.

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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 sphingomylins, 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)P_2] but inhibition by phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2], and permeation to Ca^{2+}, Fe^{2+}, and Mn^{2+}, but block by Fe^{3+}, 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)P_2 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)P_2 is present, and with the critical PI(3,5)P_2 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.

2. 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)P_2 (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.

2.1. Cell culture and transfection

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

2.2. Electrophysiology

For whole-cell recordings, recording pipettes were pulled from standard wall borosilicate tubing with filament (Sutter Instrument) to 2–3 MΩ when filled with the pipette solution containing (in mM): 135 Cs-methanesulfonate, 10 CsCl, 1 $MgCl_2$, 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 $CaCl_2$, 1 $MgCl_2$, 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 $MgCl_2$, 0.39 $CaCl_2$, 1 EGTA, 10 HEPES (pH 7.2 by KOH). For inside-out recordings, the pipettes were pulled to 1–2 MΩ when filled with Tyrode's solution and placed into the bath.

For whole-lysosome recordings, vacuolin-1 (1 μM, 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 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, and 10 2-morpholinoethanesulfonic acid (pH 4.6 by HCl) and with resistance of ~10 MΩ. 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 second. For voltage steps, 400 ms steps from −80 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 μm from the sample being recorded. The solution exchange was complete within 1–2 s. All experiments were conducted at the room temperature (22–23 °C).