Development of a manual patch-clamp assay to characterise native endo-lysosomal ion channels

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Introduction

Lysosomes are a critical component of eukaryotic cells, playing a role in degradation and recycling processes, signal transduction and extracellular secretion[I]. Ion channels expressed on the endo-lysosomal membrane are crucial in intracellular signalling and maintaining the acidic luminal pH for optimal hydrolase activity[II]. There are a number metabolic disorders, known as lysosomal storage diseases, that arise from lysosomal dysfunction[III]. Furthermore, targeting the autophagic-lysosomal pathway is a novel therapeutic strategy for clearance of toxic aggregates, which are pathological hallmarks of many neurodegenerative diseases.

Endo-lysosomal channels have been historically challenging to investigate due to their intracellular location in small-sized organelles. However, advances in lysosomal biology have developed a technique to enlarge and extract endo-lysosomes to be recorded using conventional patch-clamp methods.

We applied a refined manual patch-clamp technique to characterize endogenous endo-lysosomal ion channels in their native environment, suitable for investigating potential therapeutic agents. In the present study we focused on the activity of TRPML and TMEM175 channels, due to their respective implications in mucolipidosis type IV[IV] and Parkinson's disease[II]. Moreover, we investigated how pH differences found along the endocytic pathway can affect TRPML channel activation.

Figure 1: Endo-lysopatch technique

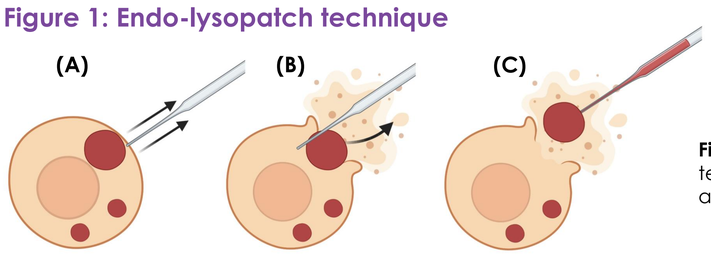


Figure 1: Schematic illustrating the technique of endo-lysosome extraction and patching[V].

Methods for endo-lysopatch

- Cells were treated with vacuolin-1 to enlarge endo-lysosomes
- Cells were briefly incubated in neutral red dye to visualise acidic vesicles within the cell **(Figure 1A)**
- A small diameter pipette was used to rupture the cell membrane and excise the endo-lysosome **(Figure 1B)**
- A fresh, fire-polished pipette was then used to patch the isolated endo-lysosome **(Figure 1C)**
- Whole endo-lysosome configuration **(Figure 2)** was achieved by applying a short duration voltage pulse
- Currents were elicited from a holding potential of 0 mV and applying a voltage ramp protocol from -100 mV to 100 mV **(Figure 3)** , applied at 0.1 Hz

Figure 2: Naïve HEK293 endo-lysosomes

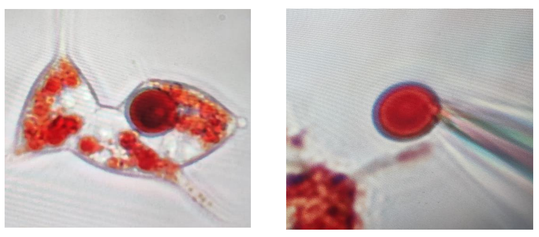
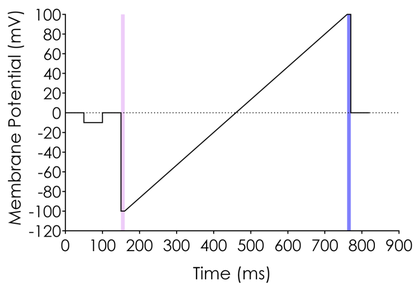


Figure 3: Voltage protocol



Whole endo-lysosome solutions:

- Intracellular (in mM): 150 Na-gluconate, 10 K-gluconate, 10 Citrate, 1 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, pH 5.5/5.0/4.4
- Extracellular (in mM): 140 K-gluconate, 10 HEPES, 3 $MgCl_2$, 10 EGTA, pH 7.2

Whole-cell solutions:

- Intracellular (in mM): 120 CsF, 15 NaCl, 10 HEPES, 10 Glucose, pH 7.2
- Extracellular (in mM): 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES/MES/Citrate, pH 7.4/6.0/5.5/5.0

TMEM175

Figure 4: Cryo-EM structure [VI]

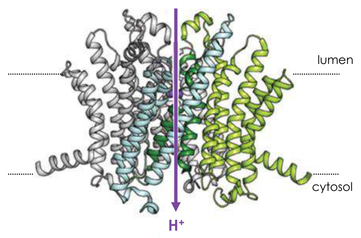
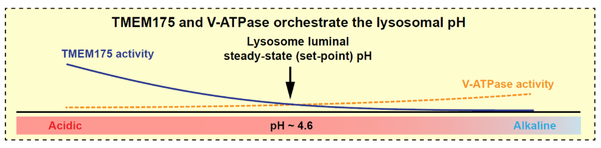


Figure 5: Regulation of lysosomal pH [II]



- TMEM175 is a homo-dimeric proton 'leak-like' channel **(Figure 4)** of the endo-lysosome system
- A regulator of the endo-lysosomal luminal pH **(Figure 5)**, primarily preventing hyper-acidification
- Channel activation by lysosomal acidic pH results in proton efflux into the cytosol
- GWAS has identified mutations in the channel in the pathogenesis of Parkinson's disease

Figure 6: TMEM175 potentiation with DCPIB

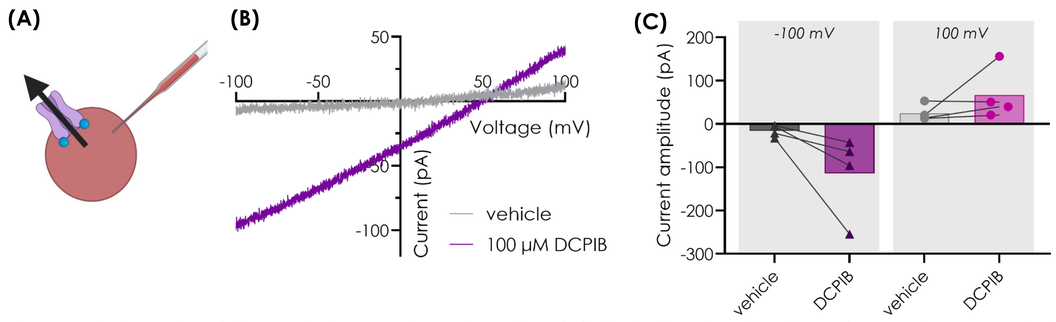


Figure 6: Schematic of the endo-lysosomal configuration (**A**), illustrating the direction of current. Representative current-voltage trace (**B**) and current distribution plots (**C**) of TMEM175, potentiated using a supramaximal concentration (100 µM) of reference agonist, DCPIB. Recorded from isolated endo-lysosomes extracted from naïve HEK293 cells.

- For representative endo-lysosome current-voltage traces, axes were inverted – a negative current indicates cation efflux from the lumen into the cytosol
- The luminal solution was set to pH 4.4 to drive proton efflux into the cytosol
- The recorded TMEM175 currents rarely exceeded -100 pA **(Figure 6)** which likely reflects low endogenous channel expression
- Therefore, a cell line stably overexpressing TMEM175 would be more suitable to develop a robust drug screening assay

TRPML1

- Ligand-gated, tetrameric Ca^{2+} release channel in the lysosome **(Figure 7 & 8)**
- Regulates lysosomal trafficking, such as fusion, fission and exocytotic events
- Activated by $PI(3,5)P_2$, a phospholipid species enriched on the lysosomal membrane
- Regulated by luminal acidic pH
- Channel mutations associated with development of mucolipidosis type IV

Figure 7: Cryo-EM structure [VII]

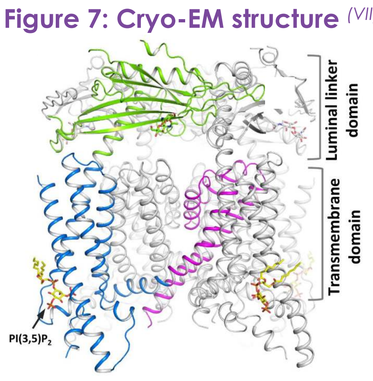


Figure 8: Transmembrane topology [VIII]

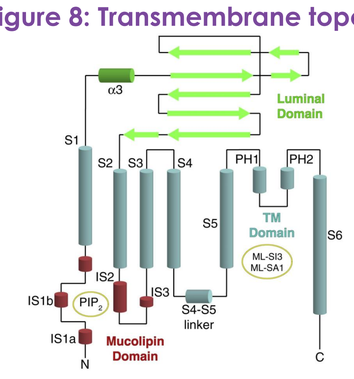


Figure 9: Effect of ML-SA1 on TRPML channels from naïve HEK293 and rat DRG

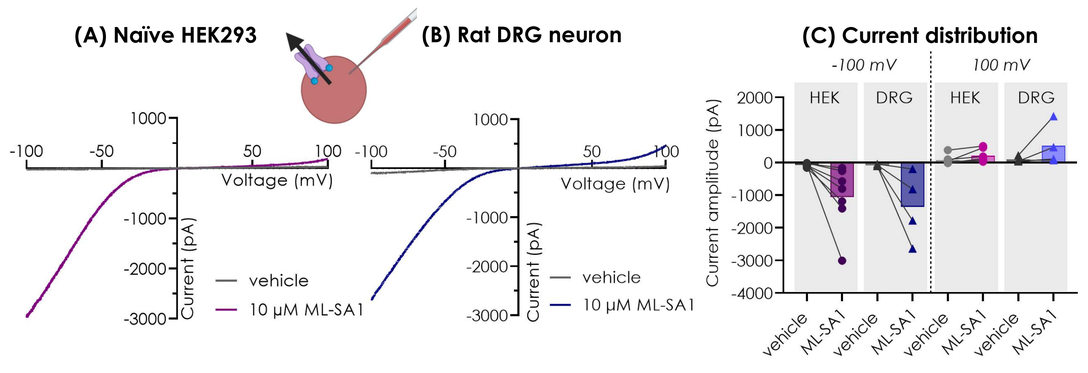


Figure 9: Representative current-voltage traces showing TRPML activation with the agonist ML-SA1 (10 µM) recorded from endo-lysosomes from (**A**) naïve HEK293 cell and (**B**) rat dorsal root ganglion (DRG) sensory neuron. Current distribution plots (**C**) elicited at -100 mV and 100 mV from both cell types.

- ML-SA1 elicited currents from naïve HEK293 and rat DRG endo-lysosomes exhibited similar profiles and amplitudes **(Figure 9)**
- These cell types are known to express all 3 types of the TRPML family, not only TRPML1

Figure 10: Mutation of organelle targeting motif [IX]

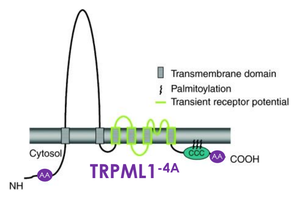
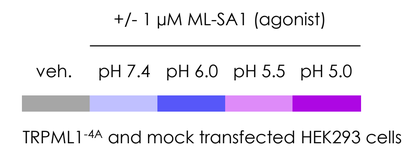


Figure 11: Whole-cell assay paradigm



- To investigate the pH sensitivity of TRPML1, we used a variant that lacks endo-lysosomal retention sequences ($TRPML1^{-4A}$), enabling the channel to express at the plasma membrane **(Figure 10)**
- The channel orientation is flipped in this condition, hence the luminal facing side on the lysosome corresponds to the extracellular facing side on the plasma membrane
- Therefore, this assay paradigm enabled multiple 'luminal' pH's to be investigated during a single whole-cell recording **(Figure 11)**
- HEK293 cells were transiently transfected with $TRPML1^{-4A}$ and recorded using conventional whole-cell patch-clamp setup **(Figure 12)**
- Transfected cells were patched in ECS pH 7.4 before switching to increasingly acidic solutions in the continuous presence of ML-SA1 (1 µM)

Figure 12: pH-dependence of $TRPML1^{-4A}$ activation

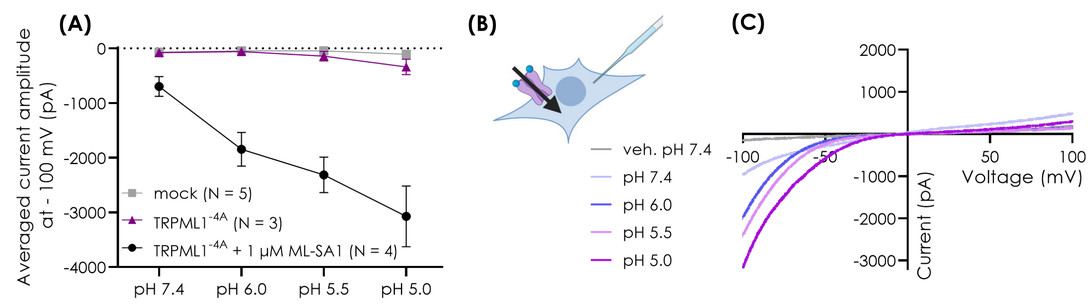


Figure 12: Mean current amplitude plot (**A**) illustrates the pH dependence of ML-SA1 induced currents of $TRPML1^{-4A}$. Data shown as mean ± S.E.M. A schematic illustrating whole-cell configuration and cation influx via $TRPML1^{-4A}$ (**B**). Representative current-voltage trace (**C**) of $TRPML1^{-4A}$ activated with ML-SA1 (1 µM) at different extracellular pH's.

- Next, the pH sensitivity of ML-SA1 activation was assessed using endo-lysosomal patch-clamp; concentration response curve assays **(Figure 13)** were recorded at different intra-luminal pH's
- Naïve HEK293 endo-lysosomes express all 3 types of TRPML channel, which are all known to be activated by ML-SA1 **(Figure 14)**

Figure 13: Whole endo-lysosome assay paradigm

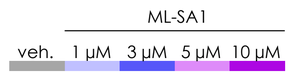


Figure 14: pH-dependence of TRPML activation in endo-lysosomes

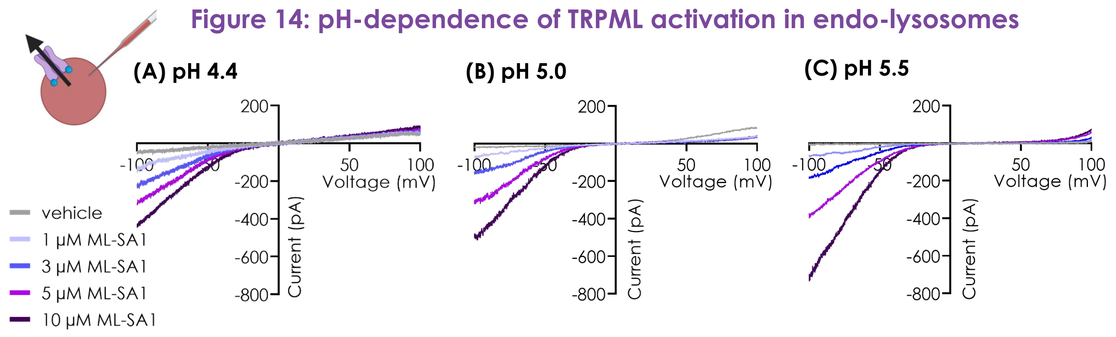


Figure 14: Representative current-voltage traces of endo-lysosomes, activated using increasing ML-SA1 concentrations (1, 3, 5 &10 µM), recorded at different luminal pH's (**A-C**). Recorded from isolated endo-lysosomes extracted from naïve HEK293 cells.

Figure 15: Potency and efficacy of ML-SA1-mediated TRPML activation at different pH's

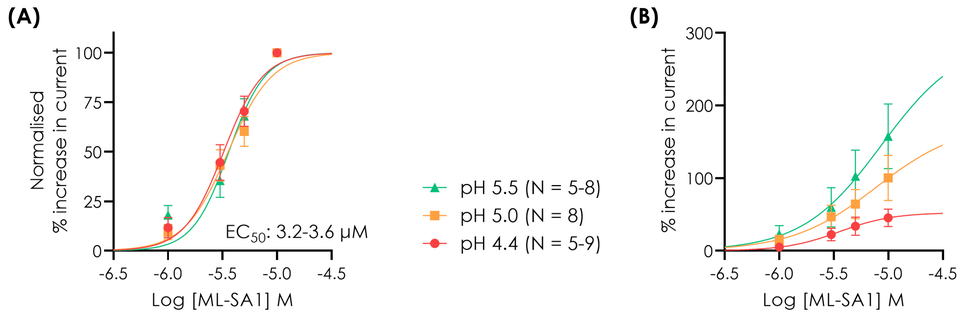


Figure 15: Concentration response curve of TRPML channels, activated using ML-SA1, recorded at different luminal pH's. Recorded from endo-lysosomes extracted from naïve HEK293 cells. Data is normalised to the maximal current at 10 µM ML-SA1 in (**A**) and % increase over vehicle baseline is shown in (**B**).

- Differences in luminal pH had no effect on the potency of ML-SA1 on TRPML channels **(Figure 15A)**
- However, changes in luminal pH influenced ML-SA1 efficacy with the least acidic condition, pH 5.5, eliciting the greatest increase in current amplitude **(Figure 15B)**
- TRPML2/3 have been reported to have different pH sensitivities to TRPML1, which may explain the difference between the endo-lysosome and whole-cell data

Summary:

- We have developed a high-quality patch-clamp technique to enable characterisation of ion channels expressed within the endo-lysosomal system
- We observe pH-dependent effects on the efficacy of ML-SA1, but not in potency when recording from endo-lysosomes
- The pH-dependent efficacy of ML-SA1 is reversed when TRPML1 is expressed at the plasma membrane for whole-cell recordings

References:

I. Luzio J. *et al.,* Nat. Rev. Mol. Cell. Biol. 2007, PMID: 17637737
II. Hu M. *et al.,* Cell 2022, PMID: 35750034
III. Xu H. & Ren D., Ann. Rev. of Phys. 2015, PMID: 25668017
IV. Sun M. *et al.,* Hum. Mol. Genet. 2000, PMID: 11030752
V. Figure made using Biorender.com
VI. Modified from Oh S. *et al.,* eLife 2020, PMID: 32228865
VII. Gan N. *et al.,* PNAS 2022, PMID: 35131932
VIII. Schmiege P. *et al.,* Structure 2021, PMID: 34171299
IX. Modified from Vergarajauregui S. & Ruestollano R., Traffic 2006, PMID: 16497227