Development of TRPML1^{-4A} assays across manual, automated patch-clamp, and fluorescence-based platforms

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Introduction

TRPML1 (transient receptor potential mucolipin subfamily 1) is a non-selective cation channel primarily localised in late endosomes and lysosomes. Channel activity is regulated by both calcium (Ca^{2+}) and pH. TRPML1 facilitates Ca^{2+} efflux from the lysosomal lumen and is, therefore, vital for vesicular trafficking processes such as exocytosis and autophagy[1].

Mutations in the gene encoding the channel protein (*MCOLN1*) results in lysosomal storage diseases, such as Mucolipidosis type IV (ML4) which presents as motor deficits, neurodegeneration and visual impairments[2].

The development and validation of electrophysiological assays to study TRPML1 is important to understand the function and pharmacology of the channel. Here, we used a TRPML1 variant that lacks the endo-lysosomal retention sequences (TRPML1^{-4A}), enabling the channel to express at the plasma membrane[3]. As such channel behaviour can be characterised by means of whole-cell patch-clamp and fluorescence-based techniques.

Aim

To develop a suite of screening assays using manual patch-clamp, automated patch-clamp and fluorescence-based platforms capable of identifying modulators of the TRPML1^{-4A} channel.

Materials and methods

Cell culture

- TRPML1 channel sequence contains two di-leucine sorting motifs that are responsible for endo-lysosomal trafficking. Mutations at these sites gives rise to the -4A mutant, which allows accumulation of TRPML1 at the plasma membrane[3].
- An inducible monoclonal human embryonic kidney 293 (HEK293) cell line expressing the TRPML1^{-4A} variant (HEK-TRPML1^{-4A}) was generated by Metrion's cell biology team.
- TRPML1 channel expression was induced by the addition of doxycycline into culture media (0.25 µg/ml to 1 µg/ml), 24 to 48 hours prior to experiments.

Compounds

- TRPML1^{-4A} currents were elicited using the agonist, ML-SA5 (Alomone Labs), stocks of which were prepared in 100% DMSO to 30 mM. Test concentrations were prepared in the appropriate solution each experimental day to a final DMSO concentration of either 0.1% or 0.3% v/v.

Patch-clamp protocol and paradigms

- Manual patch-clamp recordings were performed on a Multiclamp 700B and 1400A digitiser device (Molecular Devices) and automated patch-clamp recordings were performed on Qube 384 (Sophion).
- The following voltage protocol and assay paradigms were utilised in patch-clamp recordings:

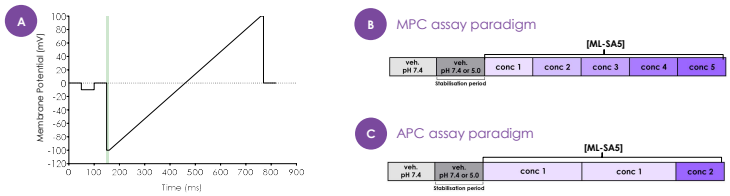

Figure 1. Currents were elicited using the voltage protocol shown in (**A**), applied at 0.1 Hz, with peak inward current amplitudes measured at -100 mV (indicated by green cursor). Assay paradigms used in the manual patch-clamp assay are depicted in (**B**). Assay paradigms used for the automated patch-clamp assay are shown in (**C**). For both MPC and APC, whole-cell conformation is obtained in pH 7.4. Inward currents were stabilised in either pH 7.4 or 5.0.

Patch-clamp solutions

The intracellular (ICS) and extracellular solutions (ECS) used in patch-clamp assays are summarised below:

ICS (in mM); CsF 120, NaCl 15, $CaCl_2$ 1, $MgCl_2$ 1, HEPES/Citric acid 10, EGTA 10, pH 7.2.
ECS for MPC (in mM); NMDG 100, NaCl 35, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 1, HEPES/Citric acid 10, Glucose 11.1, pH 7.4/5.
ECS for APC (in mM); NaCl 140, KCl 13, $CaCl_2$ 1, $MgCl_2$ 1, $BaCl_2$ 4, HEPES/MES 10, pH 7.4/5.

Fluorescence-based assay

- Fluorescence-based measurements were performed on a FLIPR Penta platform (Molecular Devices).
- Hanks buffered salt solution (HBSS + Ca^{2+}/Mg^{2+}) was supplemented with HEPES (20 mM) to pH 7.4.
- HEK-TRPML1^{-4A} cells were incubated with FLIPR calcium 6 assay dye (Molecular Devices) for 1 hour prior to the experiment.
- Fluorescent response was measured for 3 minutes after compound addition following a basal read of 20 seconds.

Results

1. Validation of the TPRML1^{-4A} stable cell line using manual patch-clamp technique

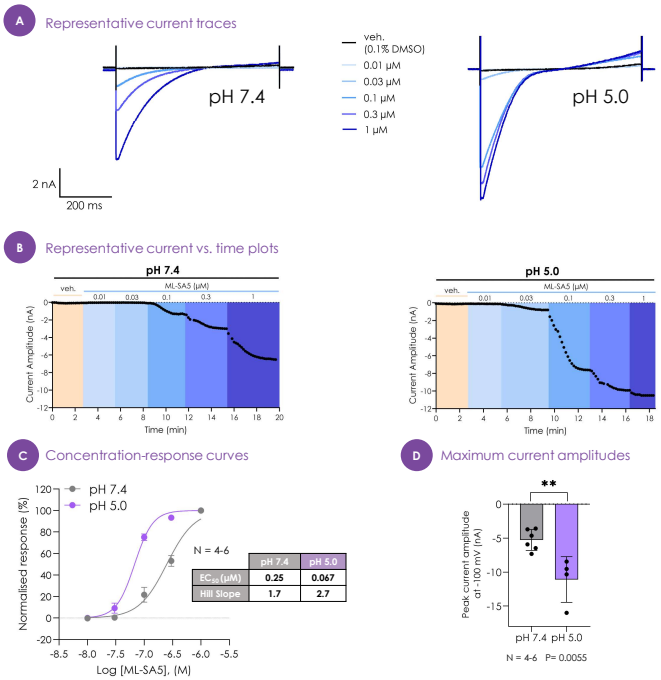

Figure 2. Comparison of ML-SA5 modulation of TRPML1^{-4A} in different pH conditions (MPC). (**A**) Representative current traces showing TRPML1^{-4A} activation by increasing concentrations of ML-SA5 (0.01 – 1 µM). (**B**) Corresponding current vs. time plots from representative traces shown in (**A**). (**C**) Concentration-response curves of TRPML1^{-4A} activation with ML-SA5 showing an increase in potency at pH 5.0 compared to pH 7.4. Data is normalised to the maximal current elicited by 1 µM ML-SA5. (**D**) Plot displaying current amplitudes for individual cells following application of 1 µM ML-SA5 in both pH conditions. TRPML1^{-4A} currents were significantly larger in pH 5.0 compared to pH 7.4 (p=0.0055). Statistical significance was determined using an unpaired Student's t-test (** p<0.01). Data represent mean ± S.D.

2. Development of an assay for use on the automated patch-clamp platform, Qube 384

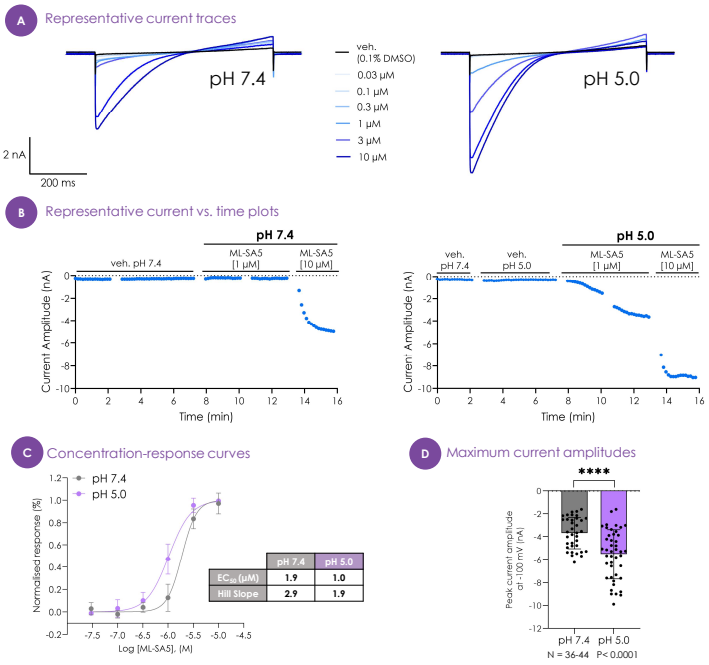

Figure 3. Comparison of ML-SA5 modulation of TRPML1^{-4A} in different pH conditions. (**A**) Representative current traces compiled from five recordings where each cell received one concentration of ML-SA5. (**B**) Representative current vs. time plot of one concentration of ML-SA5 (1 µM) followed by maximal current response elicited using 10 µM ML-SA5. (**C**) Concentration-response curves for TRPML1^{-4A} activation by ML-SA5 showed an increase in potency at pH 5.0 compared to pH 7.4. Data was normalised to maximum current activation with 10 µM. (**D**) Plot displaying maximum current amplitudes elicited with 10 µM ML-SA5 from individual cells showed a significant increase in current size at pH 5.0 compared to pH 7.4. Statistical significance was determined using an unpaired Student's t-test (**** p<0.0001). Data represent mean ± S.D.

3. Development of a fluorescence-based assay using the FLIPR platform

The reference agonist, ML-SA5 was tested at 10 concentrations (0.0003 to 10 µM) at pH 7.4.
Two assay runs were performed to ensure reproducibility between experiments.
Area under curve was calculated for each well and concentration-response curves were constructed.

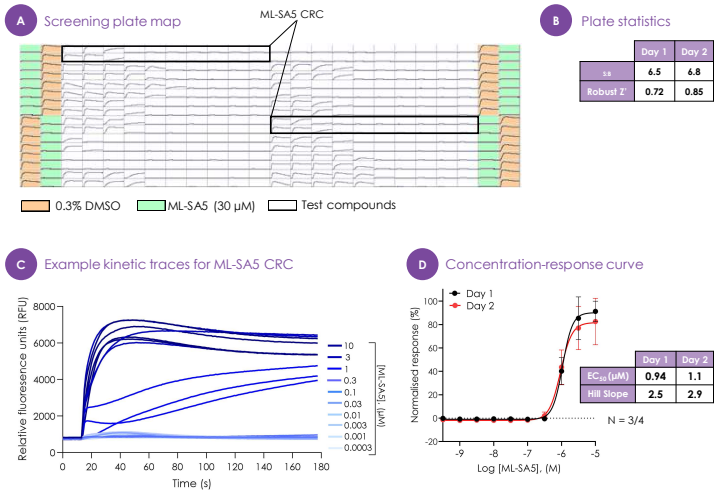

Figure 4. TRPML1^{-4A} FLIPR assay using the agonist ML-SA5. (**A**) Example screening plate consisting of assay standard (highlighted) and test compounds of unknown properties. (**B**) A summary of statistics for day 1 and day 2 plates including signal to background ratio and Z' value. (**C**) Example kinetics traces for ML-SA5 (0.0003 -10 µM) extracted from day 1 plate data. Data corresponds to the ML-SA5-containing wells indicated by the labelled boxes in (**A**). (**D**) Concentration-response curves of TRPML1^{-4A} activation by the agonist ML-SA5 in pH 7.4. Data show excellent reproducibility across two experimental days. Data represent mean ± S.D.

Conclusions

- **We successfully generated a HEK-TRPML1^{-4A} stable cell line which can be functionally characterised using electrophysiological techniques.**
- **Leveraging both patch-clamp electrophysiology and fluorescence-based assays, we were able to optimise screening services capable of studying TRPML1 modulators with excellent cross-platform correlation.**
- **The EC_{50} values we obtained for ML-SA5 were in line with literature for FLIPR[5] and Qube[6] platforms.**
- **The pH sensitivity of TRPML1 was confirmed in our patch clamp assays showing greater response to ML-SA5 in acidic pH conditions.**

References

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