

Investigation of the Ion Channels hTMEM16A/Ano1 and TRPC5 and their Modulation by Intracellular Calcium

nan]i[on

Nina Brinkwirth¹, Tom Götze¹, Markus Rapedius¹, Søren Friis¹, Andrea Brueggemann¹, Tim Strassmaier¹, Claudia Haarmann¹, Ilka Rinke¹, Marius Vogel¹, Timo Stengel¹, Johannes Stiehler¹, Michael George¹ and Niels Fertig¹

¹Nanion Technologies GmbH, Gabrielenstr. 9, 80636 Munich, Germany, contact: Info@nanion.de



Abstract

Targets

hTMEM16A/Anocamin1 is a Ca^{2+} activated Cl^- channel (CACC) which has a broad functional spectrum in processes including trans-epithelial ion transport, olfaction, photo-transduction, smooth muscle contraction, nociception, cell proliferation and control of neuronal excitability¹. The ion channel has been implicated to play a role in a number of health disorders and may be an important therapeutic target in cystic fibrosis, asthma, pain and some human cancers¹.

hTRPC5 is a non-selective cation channel expressed in many areas of the brain particularly the hippocampus, amygdala and cerebellum, amongst others². Although the physiological and patho-physiological role of hTRPC5 is not fully known, it does appear to be important in neuronal function, in particular during development where it is involved in hippocampal neurite outgrowth and growth cone morphology^{3,4}. Knockout mouse studies have also revealed that TRPC5 plays an essential role in innate fear⁵. hTRPC5 is expressed in some regions outside of the CNS including the heart⁶ where it contributes to cardiac hypertrophy in heart failure⁶.

Challenge

hTMEM16A and hTRPC5 are activated by elevated cytosolic Ca^{2+} concentrations. To initiate channel activity intracellular solution must be exchanged from a Ca^{2+} free solution to a solution containing Ca^{2+} . Some of the common instruments for automated patch clamp are limited to do an internal exchange at all. But also for the other devices it is challenging because they rely on fluoride in the internal solution to perform proper seals. Unfortunately fluoride has a strong tendency to precipitate with calcium, which makes it impossible to correctly adjust a precise Ca^{2+} concentration needed for channel activation.

Furthermore current run-down or desensitization are common problems associated with recording hTMEM16A⁷ and hTRPC5¹⁰.

Accomplishment

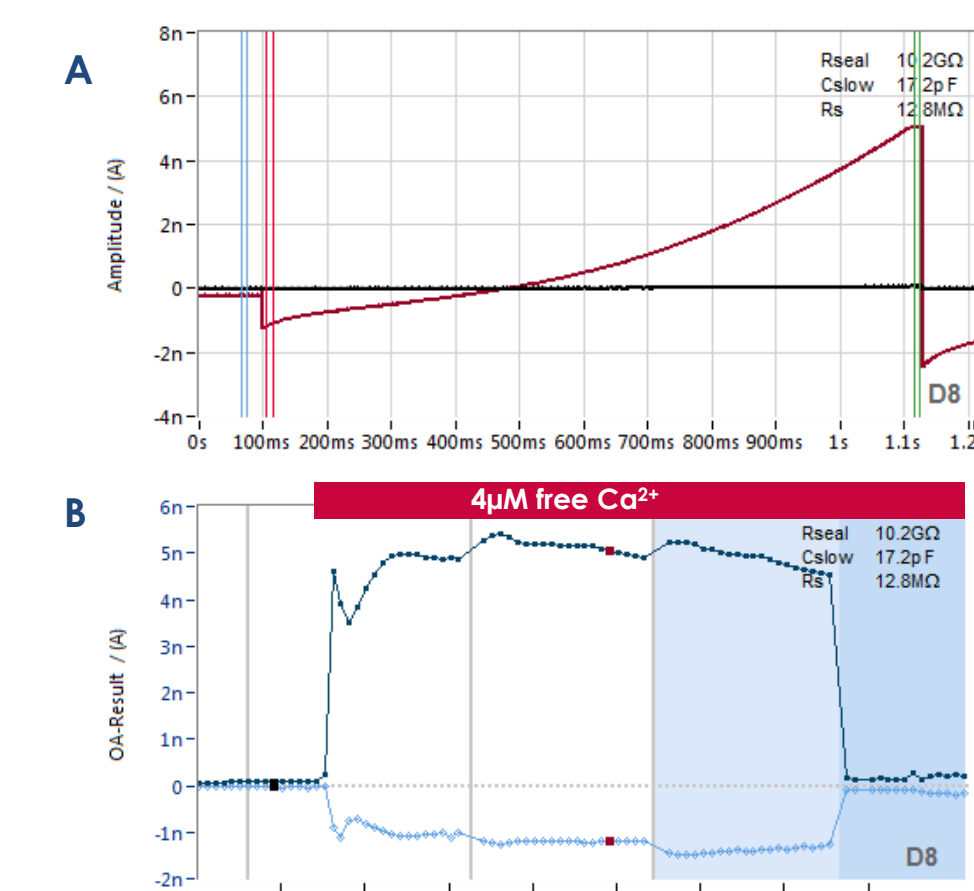
On the SyncroPatch® 384PE we developed two very reliable assays for hTMEM16A and hTRPC5 in the presence of fluoride-free intracellular solutions using the internal perfusion feature. The presented data show that the intracellular solution can be exchanged in a very robust manner to investigate Ca^{2+} sensitivity.

Besides voltage dependence and pharmacology characteristics of hTMEM16A and hTRPC5 are presented on this poster. The hTMEM16A assay was established using the PE/Felix wheatear the hTRPC5 assay was run on the PE/Biomex.

hTMEM16A/Ano1 Internal Perfusion of Ca^{2+} to Activate the Channel / Inhibition by NFA

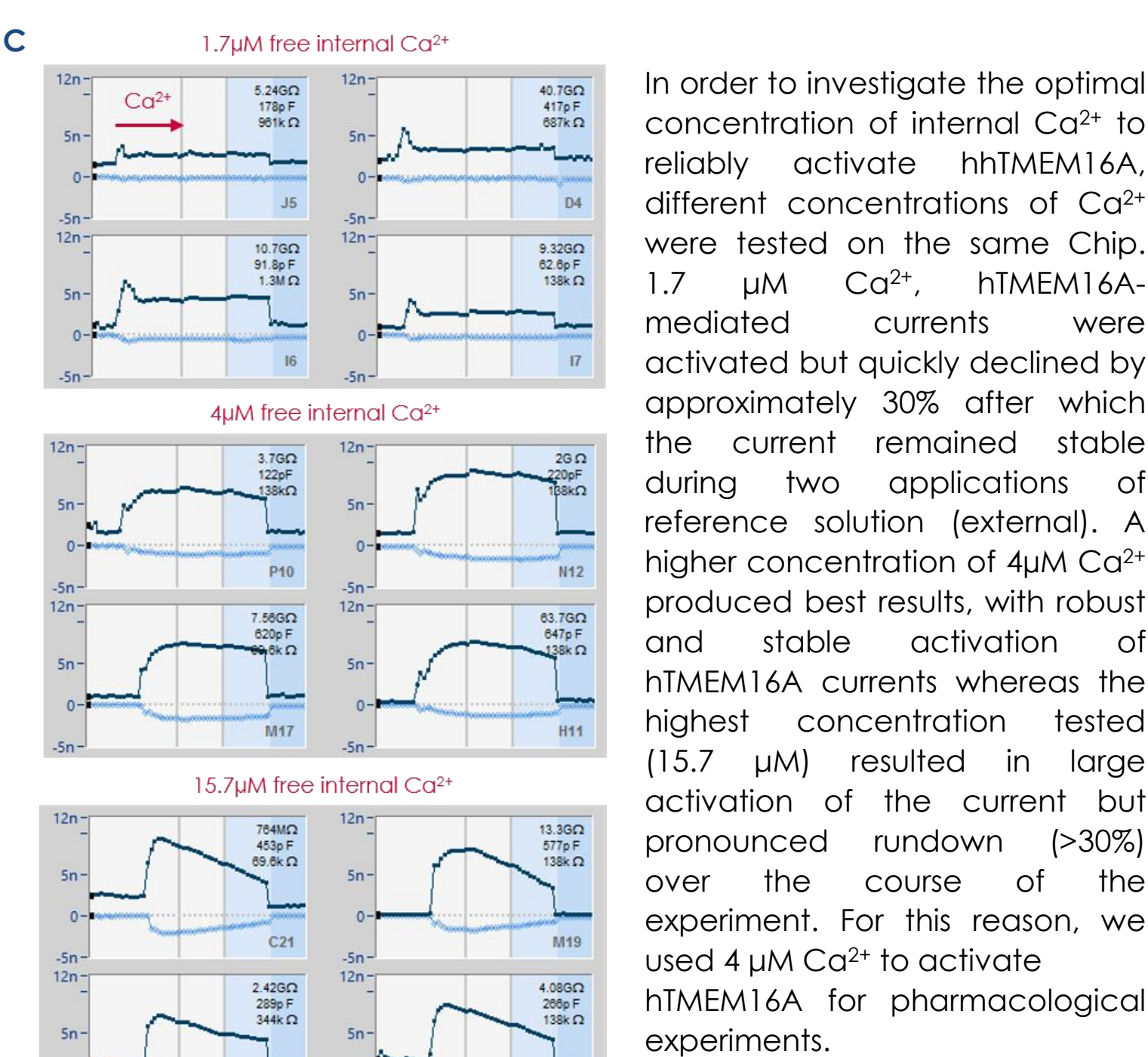
Stable current over 10 min

hTMEM16A gating is influenced by internal Ca^{2+} and membrane potential. To activate hTMEM16A the internal perfusion system was used while applying voltage ramps. Upon application of internal Ca^{2+} the current increased reaching a peak within 1-2 min after the start of perfusion. The current was stable and completely inhibited by NFA.



Activation of hTMEM16A by internal perfusion of 4 μM free Ca^{2+} .
A Row traces of an example cell to a voltage ramp from -120 mV to +60 mV in the absence (black trace) and presence (red trace) of 4 μM internal free Ca^{2+} . B Current amplitudes at +60 mV (dark blue) and -120 mV (light blue) are plotted versus time. The application of internal Ca^{2+} is indicated by the red bar. During the experiment extracellular buffer was applied twice (white region), followed by reference buffer (light blue region) and at the end a full-block concentration of 250 μM NFA was added (darker blue region).

Precise Ca^{2+} concentration is important

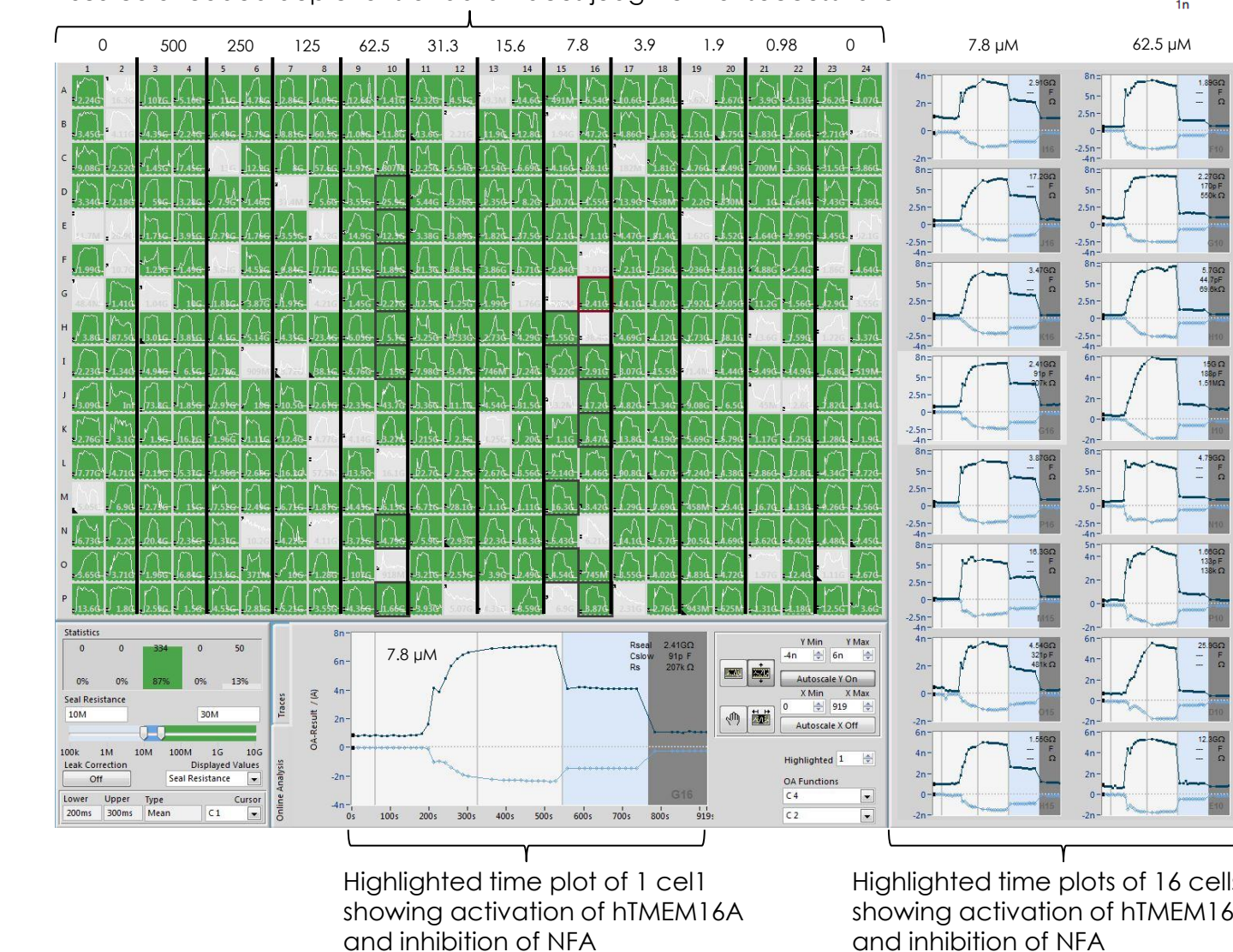


Representative Online Analysis using different concentrations of free Ca^{2+} for activation.
C hTMEM16A was activated by internal perfusion and extracellular solution was applied twice (grey lines: RefSol). The current amplitude at 100 mV (dark blue) and -100 mV (light blue) is plotted versus time. Full block with NFA was performed at the end

IC50 of NFA depends on voltage

A 10-point dose response curve of Niflumic acid (NFA) was recorded on hTMEM16A using the SyncroPatch® 384PE reaching a success rate of 87% (334 wells).

385 color coded depictions of data traces judgment of success rate

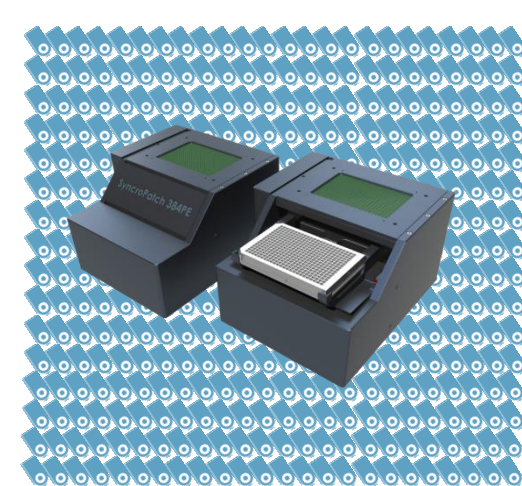


Concentration response curves of NFA on hTMEM16A at different voltages.
D Graphical user interface of the screening and data analysis software used on the SyncroPatch® 384PE. Graphs show current amplitudes following internal Ca^{2+} (4 μM) activation (white region), followed by block by different concentrations of NFA (blue region: concentrations indicated) and finally full block with 250 μM NFA (grey region). E Plot of IC50 curves depending on voltage. IC50 at -100 mV = 14.7 μM, IC50 at -20 mV = 9.6 μM (not shown), IC50 at 40 mV = 7.9 μM, IC50 at 100 mV = 5.2 μM.

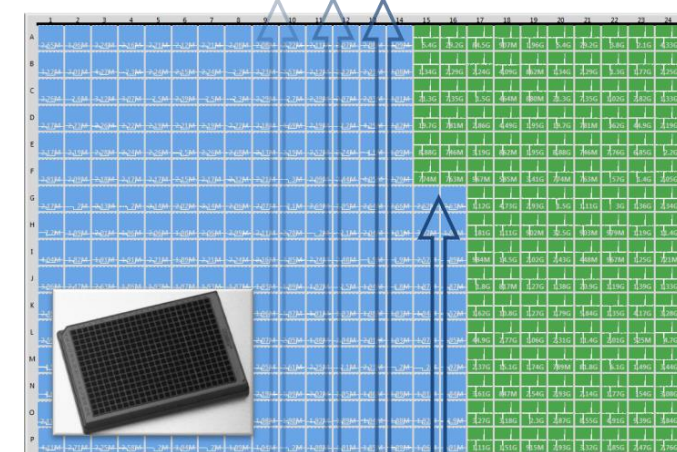
Currents were recorded using a voltage step protocol involving 4 steps to -100 mV, -20 mV, 40 mV and 100 mV each for 200 ms and with 2 s at the holding potential of -60 mV between each step. This was repeated every 15 s. The protocol allowed to analyze the IC50 of NFA at different voltages.

The concentration response curves were calculated across the plate using a full block concentration (250 μM NFA) at the end of the experiment for normalization. The IC50 values are in good agreement with those reported previously^{8,9}. NFA is less efficacious at negative potentials. This finding is consistent with published results^{8,9}.

PatchEngine HTS for Automated Patch Clamp



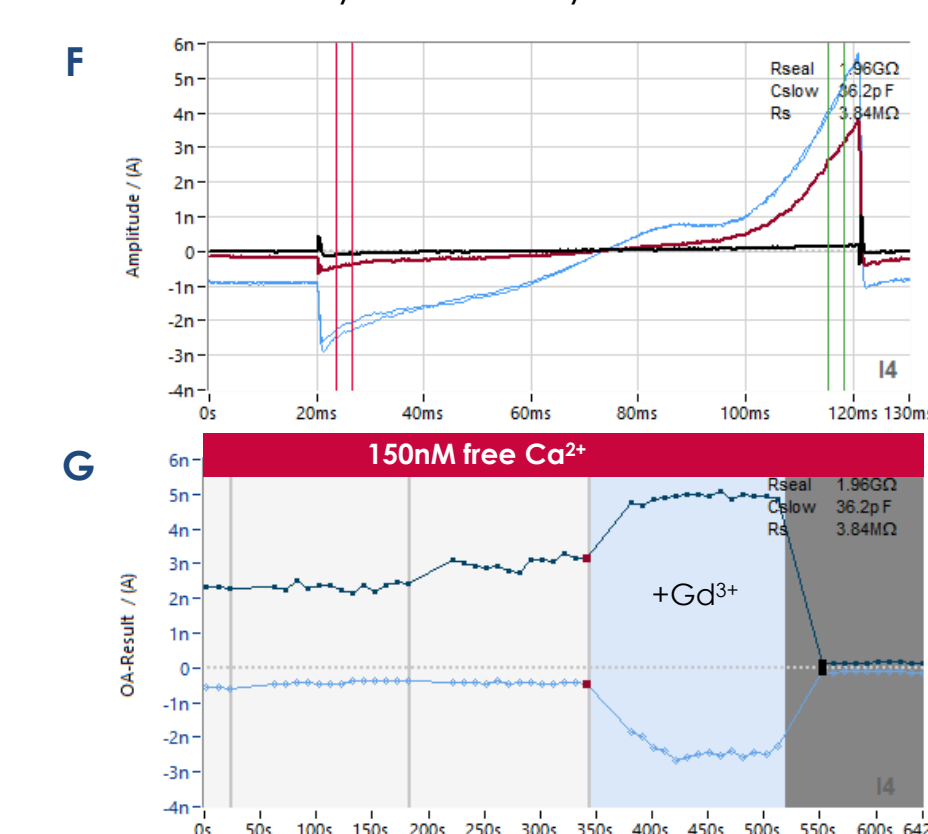
- ✓ Compatible with different liquid handler: Felix (CyBio), Biomex FX (Beckman Coulter)
- ✓ 384 amplifiers, 384 pipetting head
- ✓ High gigaseal rate on HEK, CHO, LTK, RBL, iPS and RBC
- ✓ Fast solution exchange for ligand gated channels
- ✓ Partial usage of the chips possible (32 wells per run)
- ✓ Different cell lines can be used in one run (6, 12, 24)
- ✓ Temperature controlled cell hotel (4 - 70 °C)
- ✓ Customized chips for different hole sizes, allowing optimal access and seal resistances for different cell lines
- ✓ Open design allows integration into robotic environments
- ✓ Upgradable current clamp option
- ✓ Upgradable temperature control option
- ✓ Complete, intuitive and flexible software for data acquisition and data analysis
- ✓ Internal solution exchange
 - Internal solution is filled and changed via 12 double columns
 - Up to 4 different solutions can be used in parallel
 - Multiple exchanges possible



hTRPC5 Potentiation by Gd^{3+} / Inhibition by 2-APB

TRPC5 is modulated by Gd^{3+}

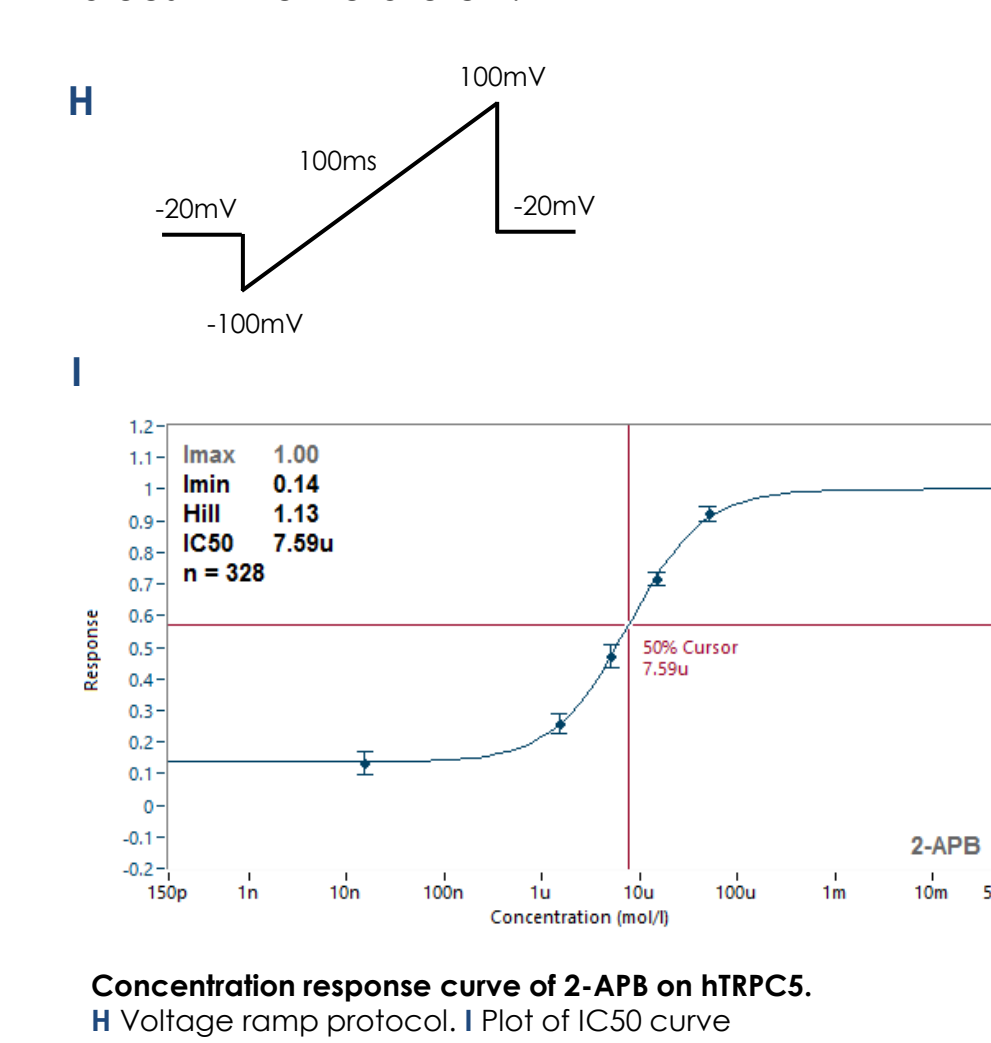
hTRPC5 is activated by cytosolic Ca^{2+} and activation is influenced by membrane potential. Furthermore hTRPC5 can be potentiated by gadolinium (Gd^{3+}). To study the modulation by Gd^{3+} hTRPC5 cells were activated by internal Ca^{2+} while applying a voltage ramp. After a while Gd^{3+} was added externally. A potentiation of the current amplitude occurred immediately, particularly for the inward current (-100 mV). This behavior stands in good agreement with published results¹⁰. Finally the current was fully blocked by 2-APB.



hTRPC5 is potentiated by Gd^{3+} .
F Exemplary raw trace to a voltage ramp from -100 mV to +100 mV activated with 150 nM Ca^{2+} in the absence (red trace) and presence (blue trace) at 100 μM Ca^{2+} . Full block was applied at the end (50 μM 2-APB, black trace). G Corresponding Online Analysis showing current amplitude at 100 mV (dark blue) and -100 mV (light blue) in presence of internal Ca^{2+} alone (white region) and plus 100 μM external Gd^{3+} (blue region). Full block with 50 μM 2-APB (grey region).

IC50 of 2-APB

hTRPC5 was activated by elevated internal Ca^{2+} and blocked by external 2-APB in a concentration-dependent manner. A ramp protocol was applied and the IC50 at 100 mV was calculated across the plate using a full block concentration of 50 μM 2-APB at the end of the experiment for normalization. Only cells which returned to baseline after full block were considered for analysis giving an IC50 of 7.59 μM and a success rate of 86% (328 wells). The IC50 stands in nice agreement with reported values in the literature¹¹.



Concentration response curve of 2-APB on hTRPC5.
H Voltage ramp protocol. I Plot of IC50 curve

Summary

- ✓ hTMEM16A (HEK293) and hTRPC5 (HEK23) were measured both successfully using the SyncroPatch® 384PE with **two different liquid handlers; the Felix (TMEM16A) and the Biomex FX (hTRPC5)**.
- ✓ The assays were stable and highly reproducible despite using **fluoride-free internal solution**.
- ✓ The **internal perfusion system** of the SyncroPatch 384PE is an indispensable feature for the investigation of channels activated by cytosolic Ca^{2+} .
- ✓ The SyncroPatch® 384PE allows to **monitor online** the current increase during internal exchange, which can be important for distinguishing compound effect from increase in leak or loss of seal and is a strong benefit for assay development.
- ✓ **Multiple internal solutions** can be **compared side by side**. This way the Ca^{2+} concentration, which delivers the most stable assay window for hTMEM16A, could be determined easily within one experiment.
- ✓ Both assays, for hTMEM16A and hTRPC5, provide a **high and robust assay window**, which allows to test inhibitors in a concentration depending manner. IC50 values were calculated across the plate using a full block at the end for a precise in-well-normalization. The IC50 values of NFA and 2-APB are in **good agreement with literature values**.
- ✓ **Excellent success rates** of 87% (hTMEM16A) and 86% (hTRPC5) were reached for IC50 experiments on both targets

➤ **In conclusion our assay development demonstrates the ability of Nanion's SyncroPatch 384PE to record challenging targets as hTMEM16A and hTRPC5 at a high throughput with excellent success rates.**

References:

- 1.] Pademonte, N. & Galletta, L.J.V., 2014. Physiol. Rev. 94: 419-45.
- 2.] Riccio, A., et al. 2002. Mol. Brain Res. 109 95-104
- 3.] Greka, A., et al. 2003. Nat. Neurosci. 6(8): 837-845
- 4.] Abramowitz, J. & Birnbaumer, L. 2009. FASEB J. 23: 297-328
- 5.] Riccio, A., et al. 2009. Cell. 137: 761-772
- 6.] Bush, E.W., et al. 2006. JBC. 281 (44): 33487-33496
- 7.] Ni, Y.-L., et al. 2014. PLoS ONE 9(1): e86734. doi:10.1371
- 8.] Bradley, E., et al. 2014. Channels. 8(4): 308 - 320.
- 9.] Liu, Y., et al. 2015. Pflugers Arch. 467(7): 1417-1430.
- 10.] Jung, S., et al. 2003. JBC. 281(44): 33487-33496.
- 11.] Xu, S.-Z., et al. 2005. Br. J. Pharmacol. 145: 405-415