

Internal perfusion of Ca^{2+} to activate TMEM16A/ANO1 on the SyncroPatch 384PE

The electrophysiology team at Nanion Technologies GmbH, Munich. Cells were kindly provided by SB Drug Discovery.



Summary

TMEM16A/Anoctamin1 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. TMEM16A 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.

TMEM16A is activated by elevated cytosolic calcium concentrations¹. In conventional patch clamp experiments, exchange of the intracellular solution to include calcium in order to initiate channel activity is challenging and typically performed using inside-out patches or by comparing the effect of internal calcium between different cells in the whole cell configuration. Furthermore, current run-down or desensitization are common problems associated with recording this ion channel².

Here we present data from HEK293 cells expressing hTMEM16A in whole cell and perforated patch mode using fluoride-free internal solution on the SyncroPatch 384PE. The data show that intracellular solution can be exchanged in a very robust manner to investigate calcium sensitivity, voltage dependence and pharmacology of the channel.

Results

CACCs such as TMEM16A are activated by cytosolic calcium and activation is influenced by membrane potential^{1,3,4}. Therefore, to activate TMEM16A the internal solution must be exchanged from a Ca^{2+} free solution to a solution containing Ca^{2+} . On the SyncroPatch 384PE the internal perfusion feature was used to activate hTMEM16A using internal application of Ca^{2+} . The current was then blocked by external application of niflumic acid (NFA; Figure 1).

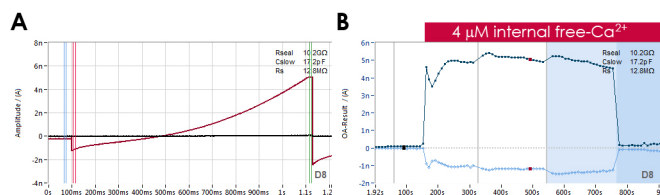


Figure 1: Activation of hTMEM16A by internal perfusion of 4 μM free- Ca^{2+} .
A Raw traces of an example cell to a voltage ramp from -120 mV to +60 mV over 1 s (intersweep holding potential -60 mV) in the absence (black trace) and presence (red trace) of 4 μM internal free- Ca^{2+} .
B Current amplitudes 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. Three minutes of baseline current was recorded prior to internal exchange. 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). The current increased upon application of internal Ca^{2+} reaching a peak within 1-2 min after the start of perfusion. The current was stable and completely inhibited by NFA. The recording was performed in the whole cell configuration using a single hole chip.

Application Note

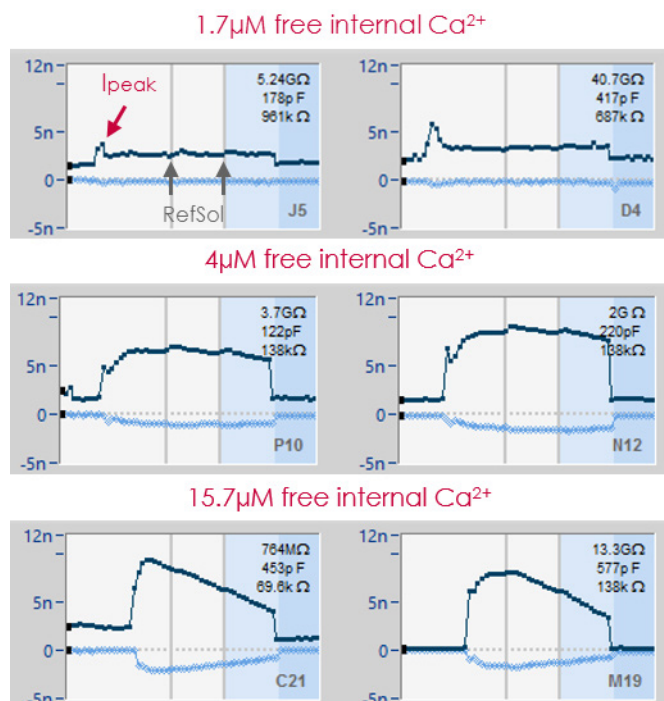


Figure 2: Representative Online Analysis using different concentrations of Ca^{2+} for activation. hTMEM16A was activated by internal perfusion and extracellular solution was applied twice (grey lines; RefSol). The current amplitude at 100 mV (dark blue) and -100 mV (light blue) is plotted versus time. Full block with NFA was performed at the end. Using 1.7 μM intracellular Ca^{2+} the channel was activated which was followed by an immediate decrease in current. The remaining smaller current was stable over time. 4 μM intracellular Ca^{2+} activated TMEM16A currents without any current decrease resulting in a robust and stable current response. At the highest concentration (15.7 μM) rundown was evident and the assay window was less stable.

In order to investigate the optimal concentration of internal Ca^{2+} to reliably activate hTMEM16A, different concentrations of Ca^{2+} were used. Figure 2 shows the current amplitudes at 100 mV (dark blue) and -100 mV (light blue) versus time for cells perfused with 1.7 μM , 4 μM or 15.7 μM internal free- Ca^{2+} . Using 1.7 μM Ca^{2+} , hTMEM16A-mediated currents were activated but quickly declined by approximately 30% after which the current remained stable during two applications of reference solution (external). A higher concentration of Ca^{2+} (4 μM) produced best results, with robust and stable activation of hTMEM16A currents whereas the highest concentration tested (15.7 μM) resulted in large activation of the current but pronounced rundown (>30%) over the course of the experiment. For this reason, we used 4 μM Ca^{2+} to activate hTMEM16A for pharmacological experiments.

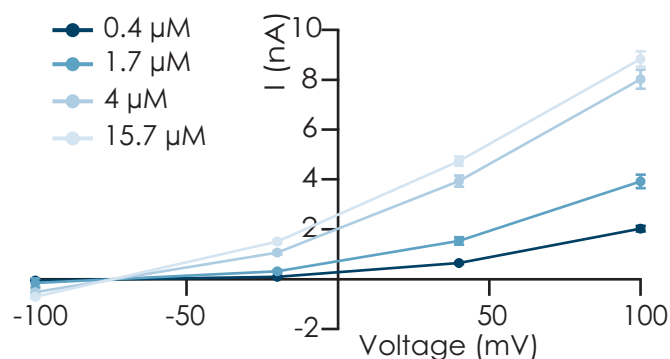


Figure 3: hTMEM16A is Ca^{2+} and voltage-dependent. Four different concentrations of Ca^{2+} were used to activate hTMEM16A in parallel on a single plate using the perfusion system of the SyncroPatch 384PE. A four step voltage protocol (-100mV, -20mV, 40mV and 100mV) was applied every 15 s. The current-voltage plot shows that current amplitude increases in response to increasing Ca^{2+} concentration and by increasing ΔV . The recording was done in perforated patch mode on a single hole chip.

Figure 3 shows that hTMEM16A activation is both Ca^{2+} and voltage-dependent as expected^{1,3,4}. In this experiment, the internal solution was exchanged from 0 Ca^{2+} to Ca^{2+} -containing solution whereby six columns of the chip received one concentration (0.4, 1.7, 4 or 15.7 μM) of Ca^{2+} and voltage was varied using a 4-step voltage protocol.

Figure 4 shows the concentration response curves of NFA at different membrane potentials. NFA is less efficacious at negative potentials. This finding is consistent with published results^{5,6}. The IC_{50} values are in good agreement with those reported previously^{5,6}.

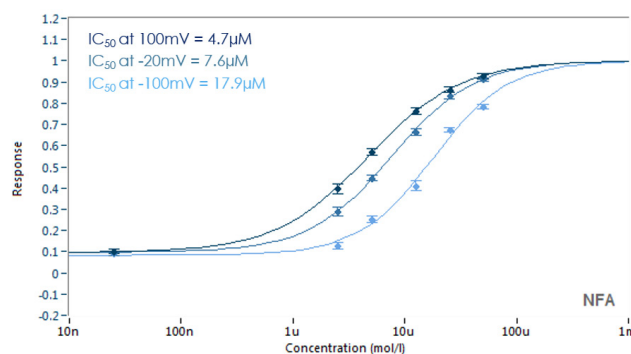
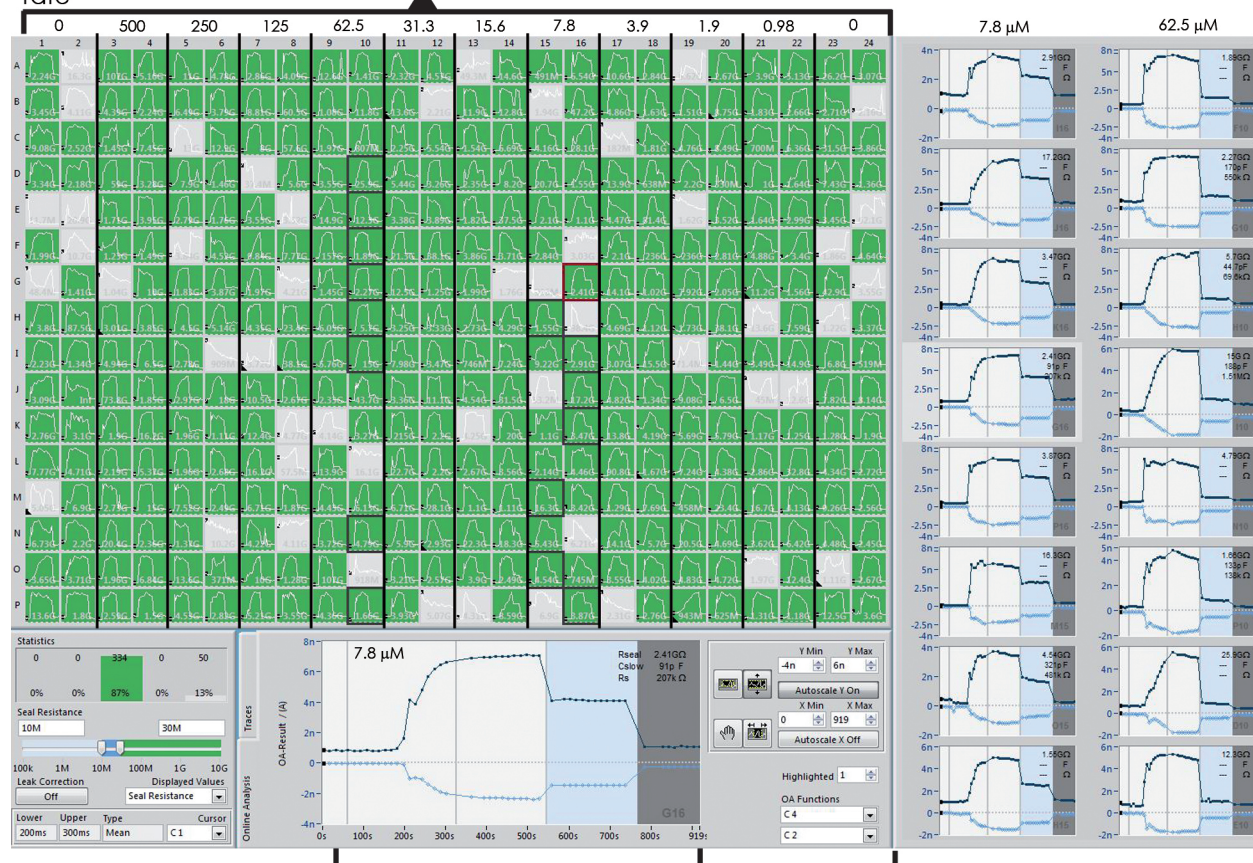


Figure 4: Concentration response curves of NFA on hTMEM16A at different voltages. The concentration response curves were calculated across the plate using a full block concentration (250 μM NFA) at the end of the experiment for normalization. IC_{50} at -100 mV = 17.9 μM , IC_{50} at -20 mV = 7.6 μM , IC_{50} at 40 mV = 6.1 μM (not shown), IC_{50} at 100 mV = 4.7 μM . The recording was performed in perforated patch mode on a single hole chip.

Application Note

384 color coded depictions of data traces eases judgement of success rate



Highlighted time plot of 1 cell showing activation of TMEM16A and inhibition by NFA

Highlighted time plots of 16 cells showing activation of TMEM16A and inhibition by NFA

Figure 5: Graphical user interface of the screening and data analysis software used on the SyncroPatch 384 L. Screenshot of depiction of online analysis data of hTMEM16A expressing HEK293 cells as recorded on one NPC-384 (single-hole) patch clamp chip. Three hundred and eighty-four small color-coded pictures as seen in the upper left part display 384 recordings. One highlighted experiment is displayed at the bottom, 16 selected experiments are displayed on the right. Graphs show current amplitudes following internal Ca^{2+} ($4 \mu\text{M}$) activation (white region), followed by block by different concentrations of NFA (blue region; concentrations indicated) and finally full block with $250 \mu\text{M}$ NFA (grey region).

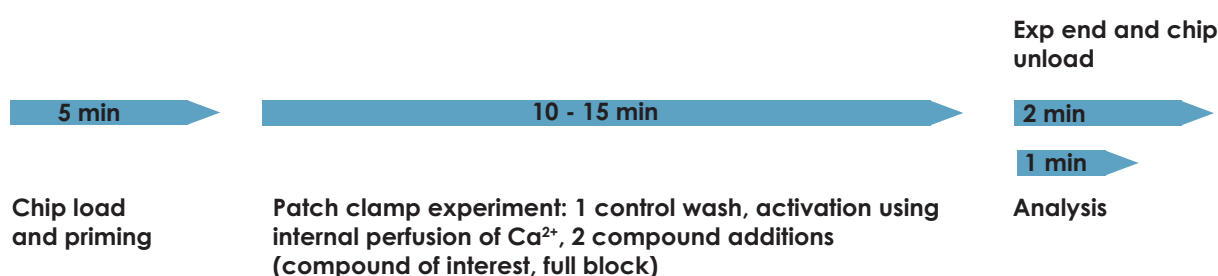


Figure 6: The completion of 1 experiment on the SyncroPatch 384 patch clamp chip (384 wells) for a single point concentration response curve plus activation of hTMEM16A with internal Ca^{2+} and full block at the end of the experiment took approximately 17-22 min.

Application Note

Figure 5 shows a screenshot of the SyncroPatch 384 software during an experiment. The user can choose whether to visualize raw traces or online analysis. In this case, online analysis is chosen and the graphs represent current amplitude plotted against time. An individual well can be highlighted to monitor the progression of the experiment. In the Online Analysis view, the time points at which solution additions have been made are indicated by vertical lines, as well as different background colors. In this case, white shows activation of hTMEM16A by internal Ca^{2+} , blue shows inhibition by different concentrations of NFA and grey shows full block with NFA.

In conclusion, hTMEM16A expressed in HEK293 cells can be recorded on the SyncroPatch 384PE using fluoride-free internal solution. The internal perfusion feature of the SyncroPatch 384PE was used to activate hTMEM16A using internal Ca^{2+} and increase in the current could be monitored online. This continuous monitoring of current amplitude is important for distinguishing compound effect from increase in leak or loss of seal and can be a benefit for assay development.

References

1. Pedemonte, N. & Galletta, L.J.V., 2014. *Physiol. Rev.* 94: 419-45.
2. Ni, Y-L., *et al.* 2014. *PLoS ONE* 9(1): e86734. doi:10.1371/journal.pone.0086734.
3. Arreola, J., *et al.* 1996. *J. Gen. Physiol.* 108: 35-47.
4. Kuruma, A. & Hartzel, H.C. 2000. *J. Gen. Physiol.* 115: 59-80.
5. Bradley, E., *et al.* 2014. *Channels*. 8(4): 308 - 320.
6. Liu, Y., *et al.* 2015. *Pflügers Arch.* 467(7): 1417-1430.

Methods

Cells

hTMEM16A expressing HEK293 cells were supplied by SB Drug Discovery, Catalogue ref: SB-HEK-Ano1.

Cell culture

Cells were cultured according to the manufacturer's recommendations and harvested according to Nanion's standard protocol using TrpLE.

Different internal solutions were used to activate hTMEM16A on a single plate to investigate Ca^{2+} dependence. The timeline of each experiment was about 17-22 minutes (start – end; Figure 6) and included wash, activation with internal Ca^{2+} , time to reach a stable current, single compound concentration application and full block, all performed on each individual well, drastically reducing the consumable cost per data point to < \$0.60.

The SyncroPatch 384PE is a high throughput and highly reliable automated patch clamp device for recording hTMEM16A currents. User-friendly software, excellent success rates, single additions or multiple additions of compound to each cell, perfusion of the internal solution and easy analysis result in reliable high quality data at an increased throughput with an economical cost per data point.

Electrophysiology

Whole cell and perforated patch clamp recordings were performed according to Nanion's standard procedure for the SyncroPatch 384PE using single hole chips and F-free internal solution. The concentration of free Ca^{2+} was estimated using the CaBuf prediction software from Guy Droogmans, Department of Physiology, KU Leuven, Belgium. Currents were recorded using a voltage ramp protocol from -120 mV to +60 mV over 1 s (intersweep holding potential -60 mV) or 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.