TMEM16A on Qube 384

A robust assay with low data spread and high success rate

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Introduction

TMEM16A (ANO1) is a Ca^{2+}-activated Cl^- channel (CaCC) that is involved in a plethora of physiological and pathophysiological conditions. The channel was suggested as a target for treatment of asthma, secretory diarrheas, and hypertension. TMEM16A is unique as its gating synergistically depends on voltage and cytosolic Ca^{2+} (Scudieri et al. 2012).
A robust and high-throughput electrophysiology assay for TMEM16A was long awaited, but progress was hampered by the fact that many automated patch clamp devices rely on the use of fluoride (F^-)in the internal solution to promote seal formation. However CaF_2 has very low solubility and the resulting precipitation limits the ability to control precisely the concentration of Ca^+ in the internal solution.

We recently developed a novel approach to test the pharmacological inhibition of TMEM16A on Qube with following characteristics:

- Consistently high success rates (>80%)
- Low run down
- High degree of pharmacological reproducibility (consistent IC50 values of multiple runs)

Results

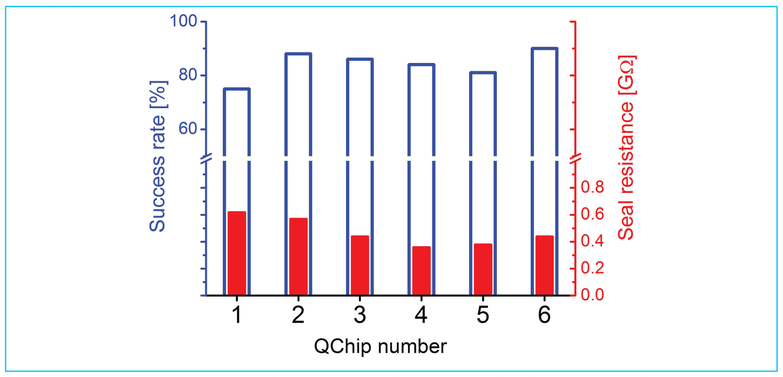

Fig. 1: Qube 384 TMEM16A assay performance: Overall success rates (blue) and whole-cell seal resistances (red) of 6 individual experiments (multi-hole QChips) performed on three different days. The filter criteria were defined as C ≥ 5 pF/cell; $I_{baseline}$ ≥ 0.5 nA/cell and $R_{membrane}$ ≥ 0.1 GΩ/cell.

These results demonstrate the high success rate and the acceptable seal resistances across multiple QChips (on different days). The average success rate of ~80% enables reliable determination of test compound potency (IC50 values) but is not high enough to allow single-point HTS. The average seal resistances of around 0.5 GΩ provide good physical stability of the seal and good sensitivity for measuring small currents.

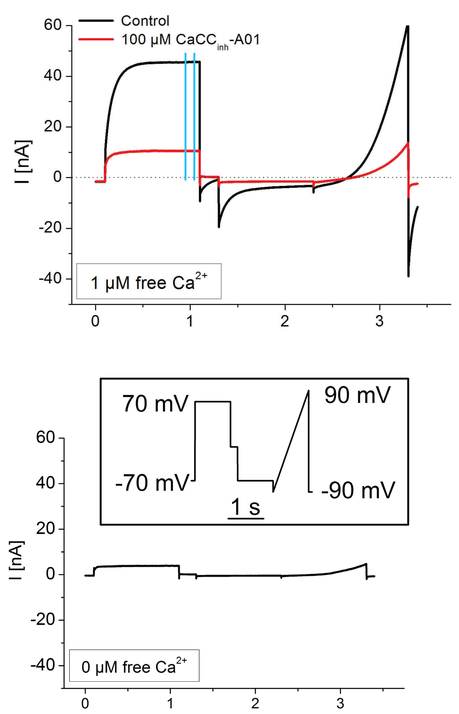

Fig. 2: Typical whole-cell TMEM16A current traces recorded using multi-hole QChips. Currents were elicited using the voltage protocol shown in the inset. A: Channels were activated in the presence of 1 µM free Ca^{2+} in the intracellular solution (black trace). Cells were subsequently exposed to the reference inhibitor, $CaCC_{inh}$-A01 at 100 µM (red trace). Steady state current was measured between the blue cursors. B: Current traces recorded in a Ca^{2+}-free internal solution.

These results illustrate the expected gating properties of TMEM16A i.e., voltage-dependent outward rectification and a dependence of activation on intracellular Ca^{2+}. In addition, the results demonstrate the sensitivity of TMEM16A to block by the known inhibitor, $CaCC_{inh}$-A01.

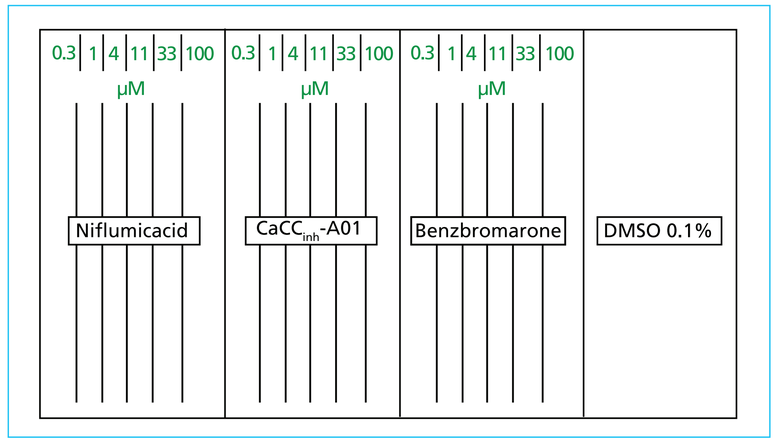

Fig. 3: Plate layout for pharmacology and stability studies. Three reference compounds were diluted to the test concentrations shown in the first 18 columns of a 384-well compound plate. The wells in the last 6 columns of the plate were filled with saline + 0.1% DMSO and were used to assess stability of the assay.

This plate layout enabled (i) multiple 6-point concentration-response curves to be generated from the current-inhibition data for each of the 3 reference TMEM16A inhibitor compounds and (ii) stability of the current in the presence of the solvent DMSO (0.1%, which is the same as the DMSO present in the test wells) to be determined.

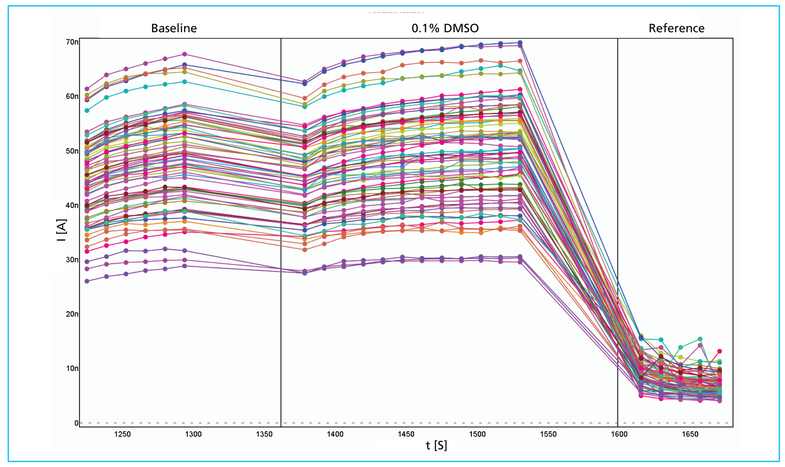

Fig. 4: I-T plot of the steady-state TMEM16A current measured at +70 mV (see Figure 2A). Each series of points represents the current measured in a single well and illustrates the stability of the current under saline-control ('baseline' period) conditions and following exposure of the cells to 0.1% DMSO (3 minutes). 100 µM $CaCC_{inh}$-A01 was applied at the end of the experiment ('reference' period) to demonstrate full-inhibition of the current.

These results demonstrate excellent stability of the TMEM16A current in saline & DMSO control conditions for at least 5 minutes as well as the complete inhibition of the current by the known TMEM16A blocker, $CaCC_{inh}$-A01 (100 µM). Such a stable assay is favorable for accurate determination of pharmacological inhibition of the channel.

Table 1: Summary current stability over time of four individual experiments (Runs 1-4). Runs 1-2 were obtained using single-hole plates and Runs 3-4 using multi-hole plates. Shown are mean ± SD of n = 79-90 cells.

	Run 1	Run 2	Run	Run 4
	Run-down [%/min]	Run-down [%/min]	Run-down [%/min]	Run-down [%/min]
0.1% DMSO	1 +/-2	-1 +/-1	1 +/-3	0 +/-2

These results demonstrate that TMEM16A displays excellent stability across multiple plates in the presence of DMSO. Excellent stability is required for accurate and confident estimation of test compounds' IC_{50} values.

References

Scudieri, P., Sondo, E., Ferrera, L., & Galietta, L. J. V. The anoctamin family: TMEM16A and TMEM16B as calcium-activated chloride channels. Experimental Physiology, 97(2), 177–183, (2012). (http://doi.org/10.1113/expphysiol.2011.058198)

Cells

HEK293 cells stably expressing hTMEM16A, kindly provided by SB Drug Discovery, were used in the Qube and QPatch experiments. The eYFP-quench experiments used a cell line that was engineered in-house at Amgen.

Fig. 5: Multiple concentration-response curves for $CaCC_{inh}$-A01 on the steady-state TMEM16A current. Each row of the compound plate shown in Figure 3 was used to generate one concentration-response curve for each compound (only $CaCC_{inh}$-A01 shown here). Data were normalized to the 'baseline' period (representing 100% current) and 'reference' period (100 µM $CaCC_{inh}$-A01 representing 100% block). Data points were fit using a Hill function to estimate IC_{50} values.

These results illustrate the reproducibility of IC_{50} determinations for $CaCC_{inh}$-A01 across different rows within a QChip.

Table 2: Summary of IC_{50} values of 3 reference compounds determined in 4 separate experiments (Runs 1-4). Runs 1-2 were obtained using single-hole QChips whereas Runs 3-4 using multi-hole QChips. Values in the table are mean ± S.D. of individual IC_{50} values generated separately from each row of the plate (format shown in Figure 3). N was between 6 & 16 for single-hole experiments and between 9 & 16 for multi-hole experiments.

	Single-hole		Multi-hole	
	Run 1	Run 2	Run 2	Run 4
	IC_{50} [µM]			
Benzbromarone	4±2	8±3	12±2	12±3
$CACC_{inh}$-A01	7±3	7±4	13±2	11±3
Niflumic acid	16±7	21±6	19±5	25±7

These results illustrate the reproducibility of TMEM16A pharmacology for 3 reference compounds across 4 separate experiments.

Table 3: Summary of IC_{50} values of 6 TMEM16A reference compounds measured using 3 different assay platforms.

eYFP assay: The Ca^{2+}-ionophore, ionomycin, was used to activate TMEM16A over-expressed in eYFP-HEK293-cells. TMEM16A-mediated influx of iodine resulted in a quenched eYFP fluorescence signal on FLIPRtetra. The IC_{50} values were determined from a 22-point titration of each reference compound.

QPatch: The inhibitory effects of each compound were assessed at the end of a 500 ms step to +100 mV with a calculated free internal Ca^{2+} concentration of 250 nM. Cells were exposed to cumulatively-increasing concentrations of test compound for 60 s, at each concentration.

Qube: Same recording conditions as in Figure 4 were used, except that the compound exposure times were 10 minutes rather than 3 minutes because some of the compounds showed relatively slow on-sets of channel block. Values shown are from 3 separate experiments using multi-hole QChips. The free internal Ca^{2+} concentration was approximately 1 µM.

			Qube		
Compound	eYFP assay [µM]	QPatch [µM]	Run 1 [µM]	Run 2 [µM]	Run 3 [µM]
MONNA	6.35	27.34	9.92	3.75	6.70
1PBC	1.55	1.15	0.42	0.47	0.98
Dichlorophen	4.37	>30	10.81	9.50	6.33
Benzbromarone	1.52	2.70	4.18	3.37	8.61
$CaCC_{inh}$-A01	8.13	9.13	8.21	6.16	12.66
$T16A_{inh}$-A01	>55.60	>30	12.79	10.85	21.18

There is very good agreement between the IC_{50} values of all 6 reference compounds in the eYFP and Qube assays. In contrast, some compounds e.g., MONNA & dichlorophen are less potent in the QPatch assay; this may be due to the short exposure times on the QPatch coupled with slow onset of action for these 2 compounds.

Conclusions

- We have established a high-throughput electrophysiology assay for TMEM16A on the automated Qube platform
- The TMEM16A assay on Qube has a high success rate (>80%) and the currents are stable, as illustrated by the low degree of rundown in saline & 0.1% DMSO conditions
- TMEM16A-mediated Cl- currents were inhibited by known channel blockers, with IC_{50} values being reproducible between separate QChips
- The potencies of 6 TMEM16A reference compounds on Qube were in good agreement with the potencies determined in a 384-well plate-based eYFP-quench assay on FLIPRtetra

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