Application Report:

TRPM8 on QPatch HTX

Cold sensitive ion channels

Pharmacological characteristics of two TRPM8 expressing cell lines, in multi-hole and single-hole mode on QPatch 16 and QPatch HTX.

AR_PUBLIC14585-9 TRPM8 on QPatch HTX

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TRPM8 on QPatch HTX

Introduction

CHO cells expressing the cold-sensitive ion channel TRPM8 were tested on the QPatch, and HEK cells expressing TRPM8 were tested on QPatch HTX. Two agonists of TRPM8 were used for EC_{50} determinations: menthol and icilin. All data presented was obtained with QPatch and analyzed with QPatch Assay Software.

Materials & Methods

Ringer's recipes

Optimized for TRPM8:

EC TRPM8, pH 7.4 (in mM): 150 NaCl, 4 KCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES

IC TRPM8, pH 7.4 (in mM): 4 NaCl, 130 CsCl, 1 $MgCl_2$, 10 HEPES, 10 BAPTA

K-based IC TRPM8, pH 7.4 (in mM): 4 NaCl, 130 KCl, 1 $MgCl_2$, 10 HEPES, 10 BAPTA or EGTA

Physiological (standard) Ringer's recipes, optimized for CHO cells:

EC 0.0.0, pH 7.2 (in mM): 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 KCl, 145 NaCl

IC 0.0.0, pH 7.2 (in mM): 5.374 $CaCl_2$, 1.75 $MgCl_2$, 10 HEPES, 120 KCl, 31.25/10 KOH/EGTA, 4 Na_2ATP

Chemicals

-(-)-Menthol (Sigma-Aldrich product # M2780) – hereafter named '–(-)-menthol'

Menthol (Sigma-Aldrich product # M2772) – hereafter named 'racemic menthol'

Icilin (Sigma-Aldrich product # I9532)

Protocols

Whole-cell protocol

The protocol used for obtaining gigaseal and whole-cell configuration on the QPatch was optimized from a Sophion standard protocol.

Voltage protocol

Three general voltage protocols were used (the voltage protocol can be seen in all raw data plots in the following figures):

1) 100 ms ramp from -80 mV to +80 mV (several different Vhold was tried)
2) Standard holding potential (-50 mV)
3) IV protocol – from a holding potential of -100 mV the potential was stepped to -120 mV to 200 mV with 20 mV increments for 400 ms.

TRPM8 on QPatch HTX

Application protocol

Generally a four to seven point dose-response application protocol was used. First saline solution was applied to the cell for a baseline response and then the compound was added, lowest concentration first.

Cell culture

Cells were grown according to the Sophion SOP for CHO-TRPM8 cells. Cell growth medium was Hams F12 supplemented with 10 % Foetal bovine serum.

Cells were harvested with trypsin and resuspended in standard CHO-SFM supplemented with 10 mM HEPES, 0.04 mg/ml soy bean trypsin inhibitor and 10 g/ml Penicillin/Streptomycin. The final cell density was approximately 3-5 mio. cells/ml.

Results

The IV relationship of the TRPM8 ion channel as obtained on the QPatch is shown in Figure 1. The TRPM8 channel shows a strong outward current at potentials above 50 mV. The outward current is present at potentials down to 0 mV when menthol is present (in the case shown in Figure 1, racemic menthol was used). This is in good accordance with what has been presented in the literature; see e.g. Hui et al, 2005; Benedikt et al., 2007, McKemy et al, 2002 and Voets et al., 2007.

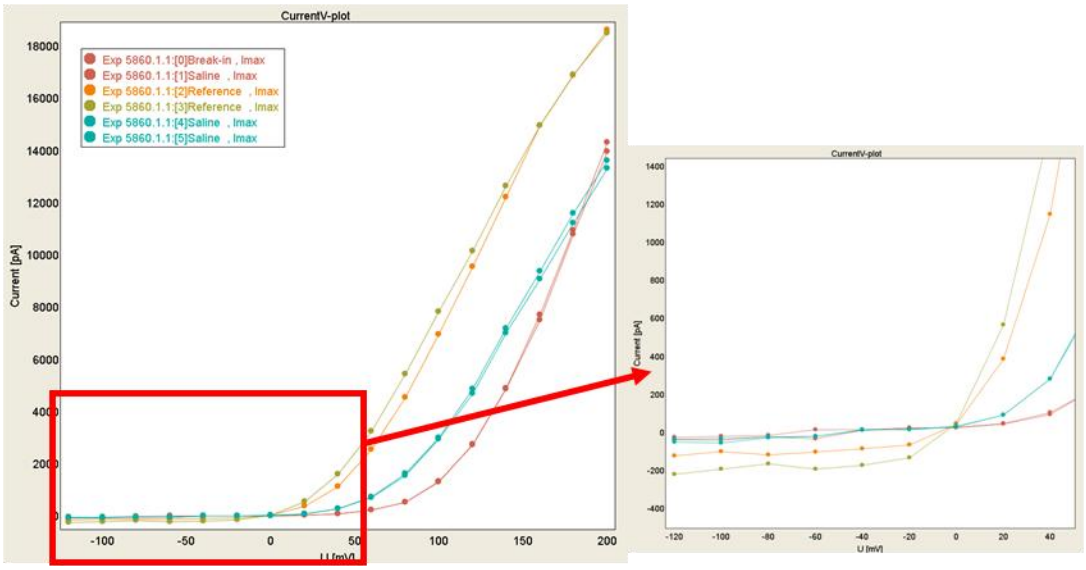

Figure 1. IV plot of TRPM8 response to saline and racemic menthol ('reference'). To the right is shown an enlargement of the response at negative potentials.

Furthermore, the TRPM8 channel displays a small inward current in response to racemic menthol at negative potentials (Figure 1, insert). This inward current is a lot more pronounced when using –(-)-menthol (data not shown).

Racemic menthol

Concentration-response relationships were obtained using the racemic menthol, with a ramp voltage protocol For these experiments, a modified version of the TRPM8 Ringer's solutions was used, where the intracellular Ringer contains potassium instead of cesium, and EGTA instead of BAPTA (see 'Ringer's Recipes').

Figure 2, Figure 3 and Figure 4 show representative raw traces, IT plot and Hill fit, respectively, obtained with the racemic menthol. Interestingly, it can be seen that this menthol does not elicit much current at negative potentials.

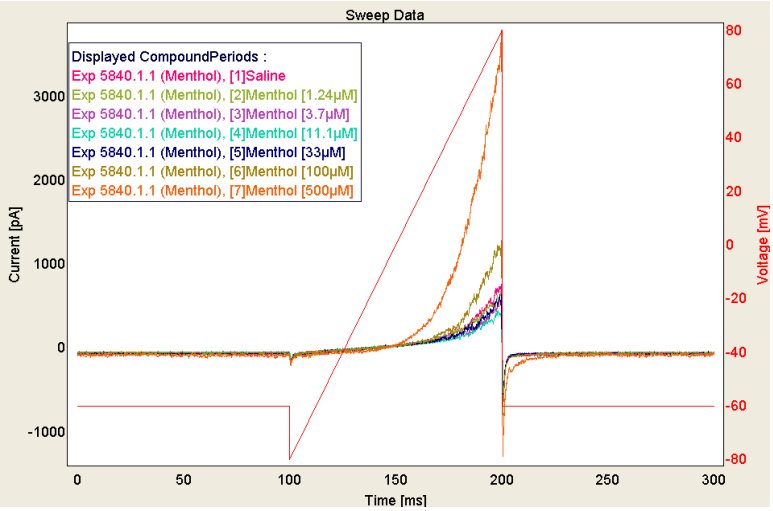

Figure 2. Representative raw current traces of the response elicited by a voltage ramp from -80 to +80 mV, when subjected to increasing concentrations of racemic menthol.

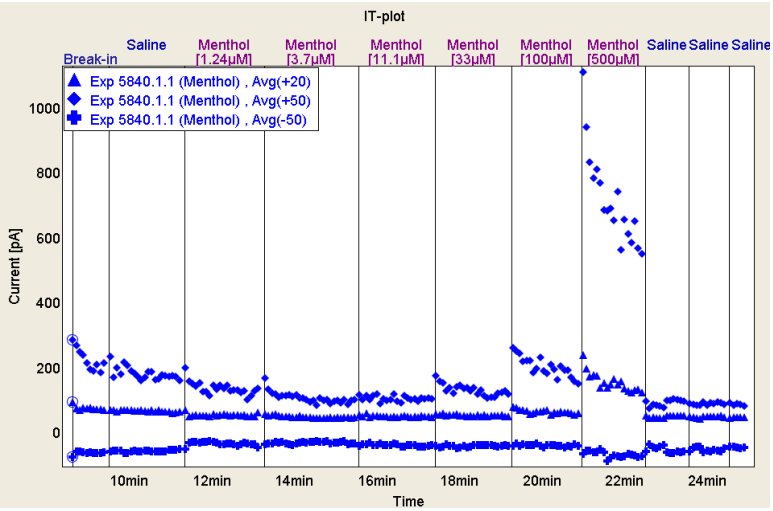

Figure 3. Representative IT-plot of the current elicited during the ramp (shown in Figure 2) at +50 mV (◇), +20 mV (Δ) and -50 mV (+), in response to increasing concentrations of racemic menthol.

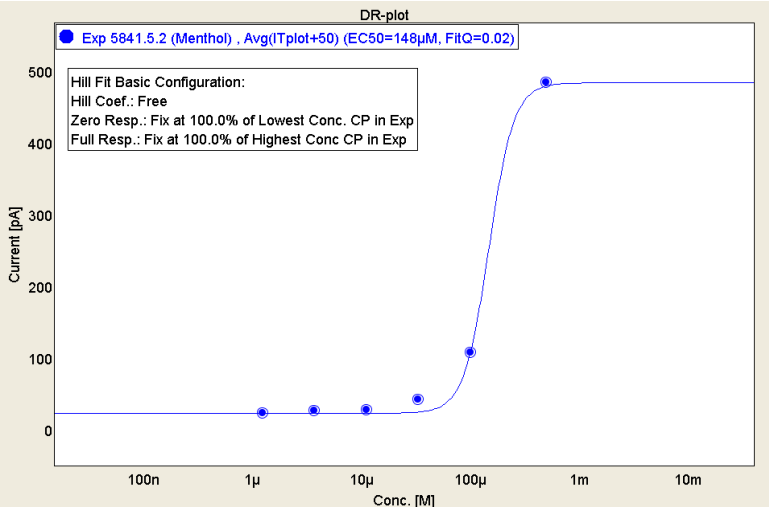

Figure 4. Representative Hill fit of the concentration-response relationship to racemic menthol (at +50 mV).

The EC_{50} values obtained with racemic menthol in K-Ringer can be seen in Figure 5. Please note that the EC_{50} estimated at -50 mV is very uncertain given that many cells exhibit little to no response to the racemic menthol at this potential.

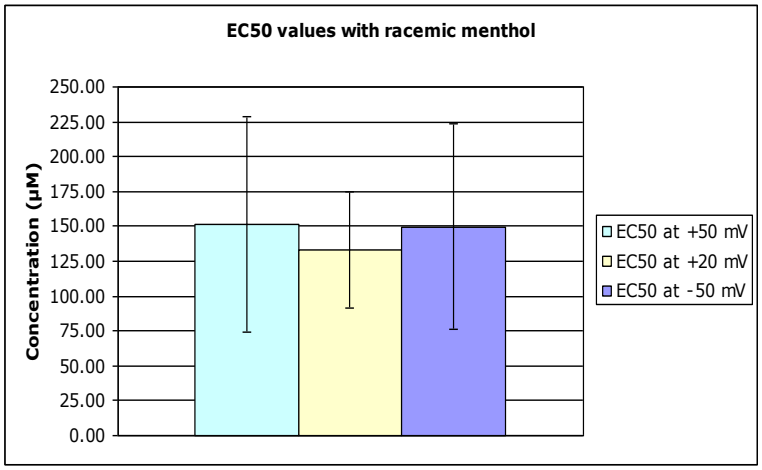

Figure 5. Graph showing EC_{50} ± SD values obtained with racemic menthol at three different potentials: +50 mV, +20 mV and -50 mV (n=30).

Because of concern that the use of potassium-based Ringer (instead of cesium) opens up the possibility of current from endogenous potassium channels, control experiments were conducted using the same experimental parameters as for menthol dose-response experiments, but on non-transfected CHO cells. The resulting current traces can be seen in Figure 6. This shows a slight inward current (<50 pA) elicited at approximately -20 mV. With regard to the experiments on TRPM8 channels, this is a negligible current.

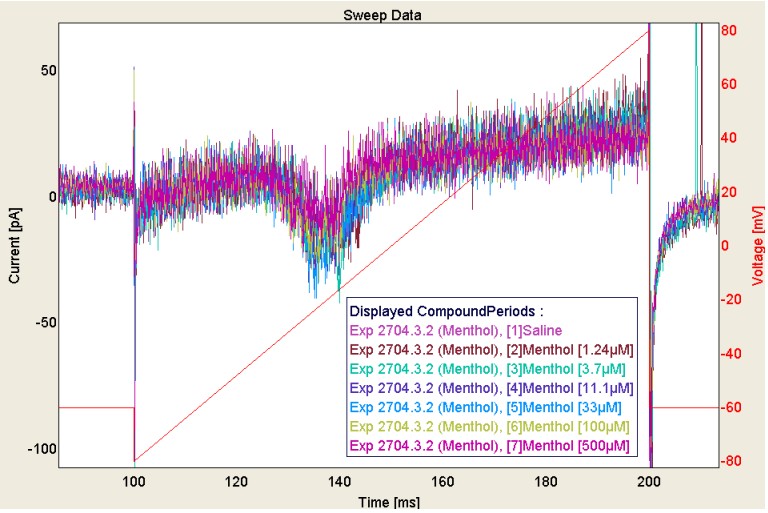

Figure 6. Sweep data from experiment conducted on non-transfected CHO cells. The experimental parameters were similar to a dose-response experiment conducted on CHO-TRPM8 cells.

-(-)-menthol

The Cs-based TRPM8 Ringer was also used for experiments. These experiments were predominantly EC_{50} determinations of -(-)-menthol and icilin.

An example of an IT plot with this menthol is shown in Figure 7. When you compare that to Figure 3 it is evident that this -(-)-menthol produces a much more pronounced inward current at negative potentials than the racemic menthol does.

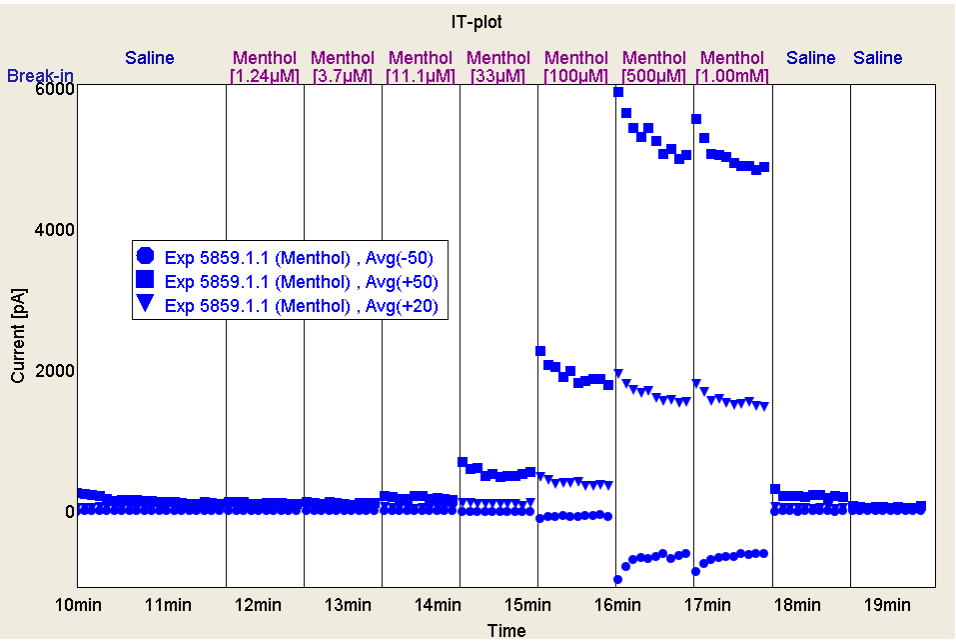

Figure 7. Representative IT-plot of the current elicited during the ramp (shown in Figure 2) at +50 mV (□), +20 mV (Δ) and -50 mV (O), in response to increasing concentrations of –(-)-menthol.

Examples of Hill fits of this concentration-response relationship at three different potentials can be seen in Figure 8, and an overview of the resulting EC_{50} estimations is shown in Figure 9. The obtained EC_{50} is in good accordance with what has been described in the literature (see e.g. Andersson et al., 2004).

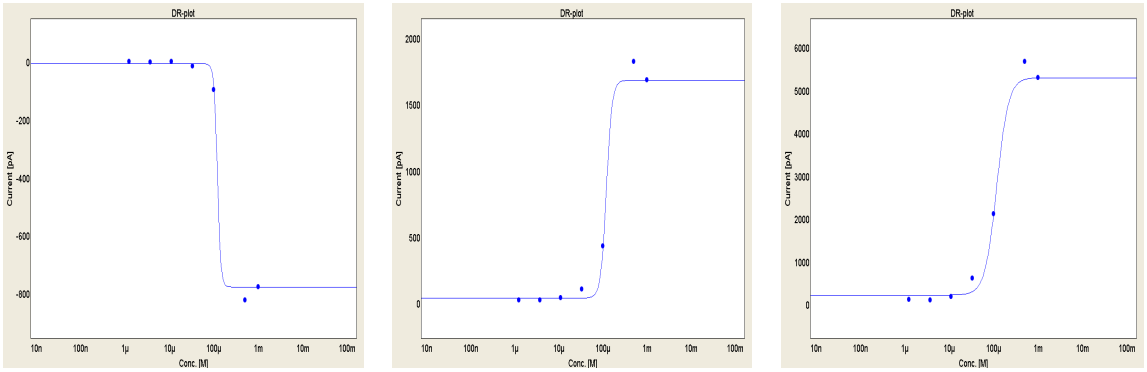

Figure 8. Representative Hill fit of the concentration-response relationship to –(-)-menthol. From the left: current level at -50 mV, +20 mV and +50 mV, respectively.

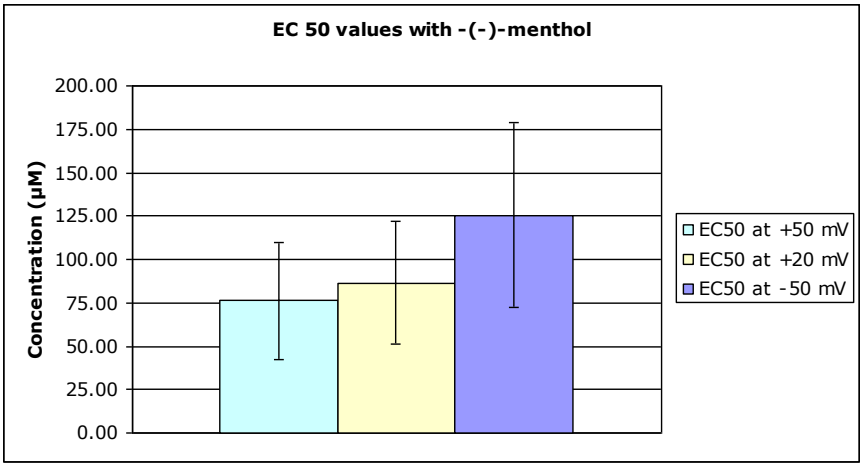

Figure 9. Graph showing EC_{50} ± SD values obtained with –(-)-menthol at three different potentials: +50 mV, +20 mV and -50 mV (n=22).

Icilin Response

Obtaining a response to icilin with TRPM8 can be a bit tricky, because it requires very strict control of free intracellular Ca^{2+} (see e.g. Chuang et al., 2004). Furthermore, the response is hard to come by, because TRPM8 exhibits very strong Ca^{2+}-dependent desensitization, which obscures the response to icilin (Chuang et al., 2004). Therefore a Ringer buffered with BAPTA was used ('IC TRPM8') for these experiments.

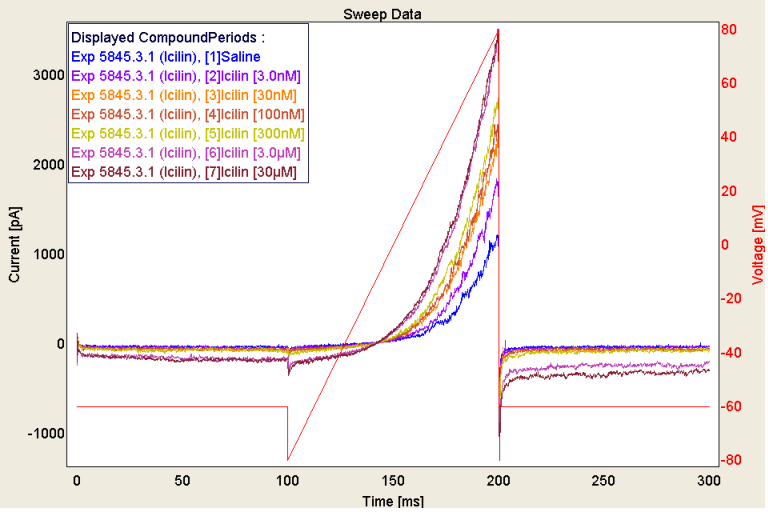

Figure 10. Raw current traces of the response elicited by a voltage ramp from -80 to +80 mV, when subjected to increasing concentrations of icilin.

Figure 10, Figure 11 and Figure 12 show the raw data, IT plot and Hill fit, respectively, for the icilin response. The EC_{50} value was in this case estimated to be 70 nM, which is in good accordance with literature values reported of approximately 100 nM (see e.g. Andersson et al., 2004).

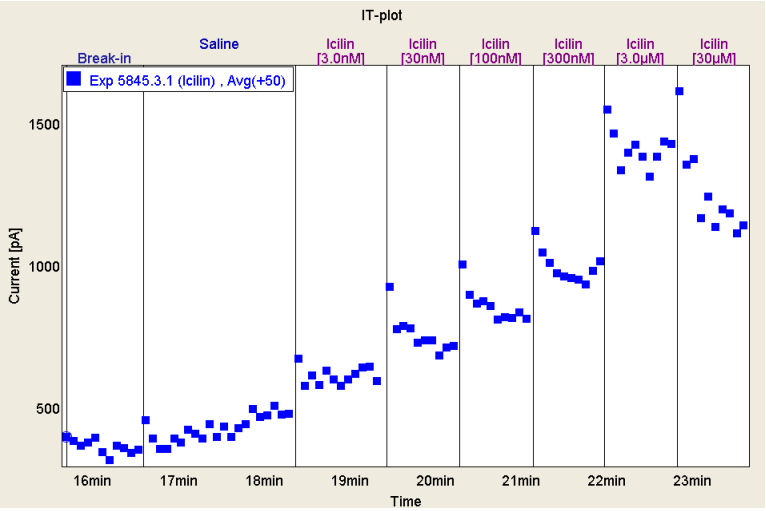

Figure 11. IT-plot of the current elicited during the ramp (shown in Figure 10) at +50 mV in response to increasing concentrations of icilin.

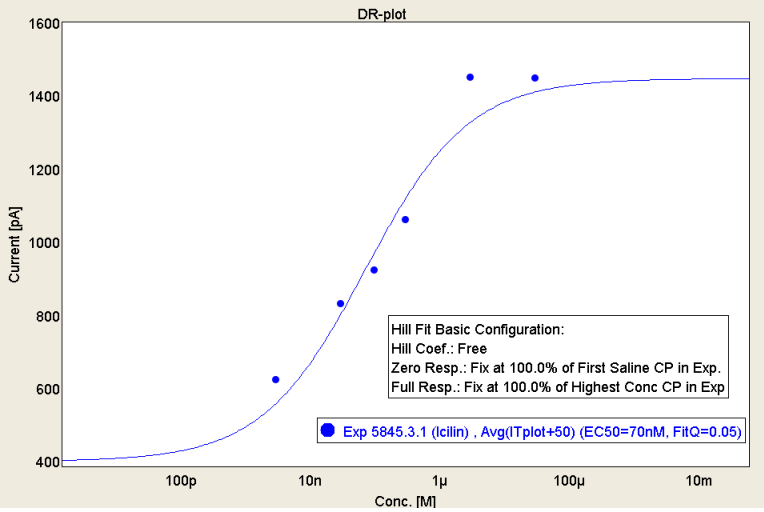

Figure 12. Hill fit of the concentration-response relationship to icilin (for the highest current amplitude elicited at +50 mV). EC_{50} was estimated to be 70 nM.

Desensitization of the TRPM8 channel was very evident when experiments were run with a stable holding potential of -50 mV to elicit menthol-evoked negative current. In Figure 13 the response elicited upon repeated stimulation of the ion channel with –(-)-menthol is shown. There was at least two minutes between each stimulation, and up to ten washes were used to remove menthol again. Nevertheless the TRPM8 channel desensitized over time, such that the amplitude of the response was not reproducible.

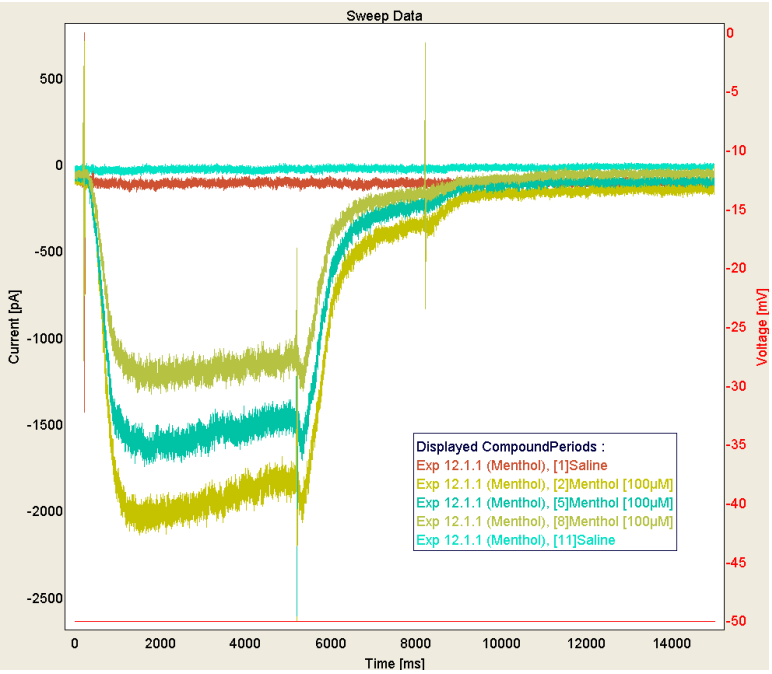

Figure 13. Representative example of 3 consecutive -(-)-Menthol-evoked current traces, obtained at a holding potential of -50 mV.

Success rates & Performance

Generally the CHO-TRPM8 cell line performed very well on the QPatch, with high quality seals and long whole-cell lifetimes. The Ringer's solution used has a large impact on the succes rates, however. Figure 14 gives an overview of the succes rate obtained with three different Ringer pairs: IC0.0.0/EC0.0.0, EC TRPM8/IC TRPM8, and EC TRPM8 with K-based IC TRPM8.

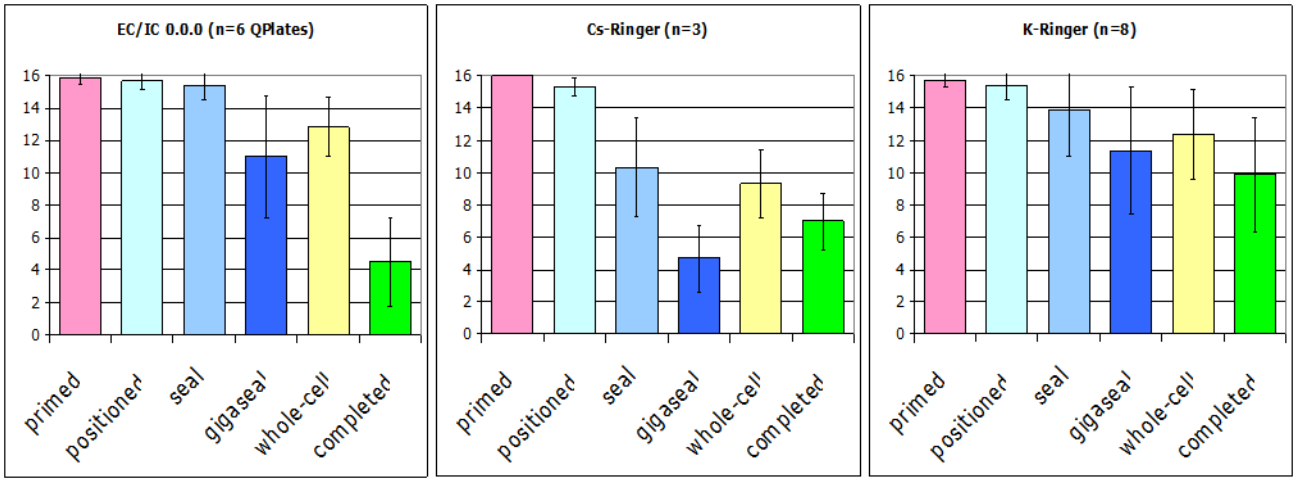

Figure 14. Succes rates ±SD of CHO-TRPM8 cells with three different Ringer pairs: IC0.0.0/EC0.0.0, TRPM8 EC/IC, and EC TRPM8 with K-based IC TRPM8. Each step in the protocol on the QPatch is considered here, with the absolute number of sites on the y-axis (experiments were conducted on a QPatch 16): no of primed sites on the QPlate, no of sites with cell positioning, no of sites with seals >100 MΩ, number of sites with real gigaseal (>1 GΩ), number of sites that have obtained whole-cell configuration, and no of completed experiments.

As can be seen, the CHO-TRPM8 cells performed best in the Ringer where cesium had been substituted for potassium. We experienced some problems when making the cesium-based Ringer, in that it precipitated with the chelating agent (EGTA or BAPTA). This is most likely the cause of the low sealing rates obtained in experiments with this Ringer.

Figure 15 shows an overview of the performance for one representative QPlate, taken from the QPatch Assay Software.

QPlate '00465535036977'

Used in job: #5852 - MKJ_TRPM8 DR Sanofi-Aventis short (5p)_notXL
Start of use: 2007-09-06 13:25:12

Pos.	Primed	Cell attached	Seal	Whole-cell	R chip [MΩ]	R seal [MΩ]	R whole-cell [MΩ]	WC duration [sec]	Completed exp.
A1	✓	✓			1.74	63.3	0.0	0	0
B1	✓	✓	✓	✓	1.81	1791.9	738.7	601	1
C1	✓	✓	✓	✓	1.80	2903.1	1039.0	582	1
D1	✓	✓	✓	✓	1.84	242.8	948.8	570	1
E1	✓	✓	✓		1.77	1650.1	0.0	0	0
F1	✓	✓	✓	✓	1.85	2538.7	1699.1	598	1
G1	✓	✓	✓	✓	1.81	4044.9	3257.6	596	1
H1	✓	✓	✓	✓	1.79	607.9	217.0	104	0
A2	✓	✓	✓	✓	1.72	3527.5	438.8	586	1
B2	✓	✓	✓	✓	1.72	1386.5	133.8	557	1
C2	✓	✓	✓	✓	1.71	1262.4	2797.6	582	1
D2	✓	✓	✓	✓	1.82	1361.8	1309.2	590	1
E2	✓	✓	✓		1.81	133.9	0.0	0	0
F2	✓	✓	✓	✓	1.79	2464.9	1616.5	588	1
G2	✓	✓			1.79	54.3	0.0	0	0
H2	✓	✓			1.77	93.0	0.0	0	0
Total	16	16	13	11					10
Success rate	100 %	100 %	81 %	69 %					

Figure 15. Representative QPlate overview taken from the QPatch Assay Software showing number of primed sites, cell attachments, seals and wholecells, and the seal resistances measured at priming (R chip), gigaseal formation (R seal) and whole-cell (R whole-cell).

Results – HEK-hTRPM8

The HEK-hTRPM8 cell line was tested for comparison of the single-hole and multi-hole technology on QPatch X. The multi-hole technology allows recordings of ten patched cells in parallel. Because the multi-hole QPlate is based on the same silicon chip technology of the single-hole QPlate, the data is of the same high quality.

Figure 16 and 17 show data from a representative dose-response experiment with the agonist -/-menthol and the antagonist capsazepine, respectively, in single-hole and multi-hole mode. Figure 18 summarizes the resulting EC/IC_{50}.

At very high agonist/menthol concentrations (>100 µM) the ion channel response is saturated for HEK-hTRPM8, and even decreases in most cases. In the CHO-TRPM8 cell line, the ion channel does not saturate until the menthol concentration reaches 500 µM. The reason for this difference is unknown.

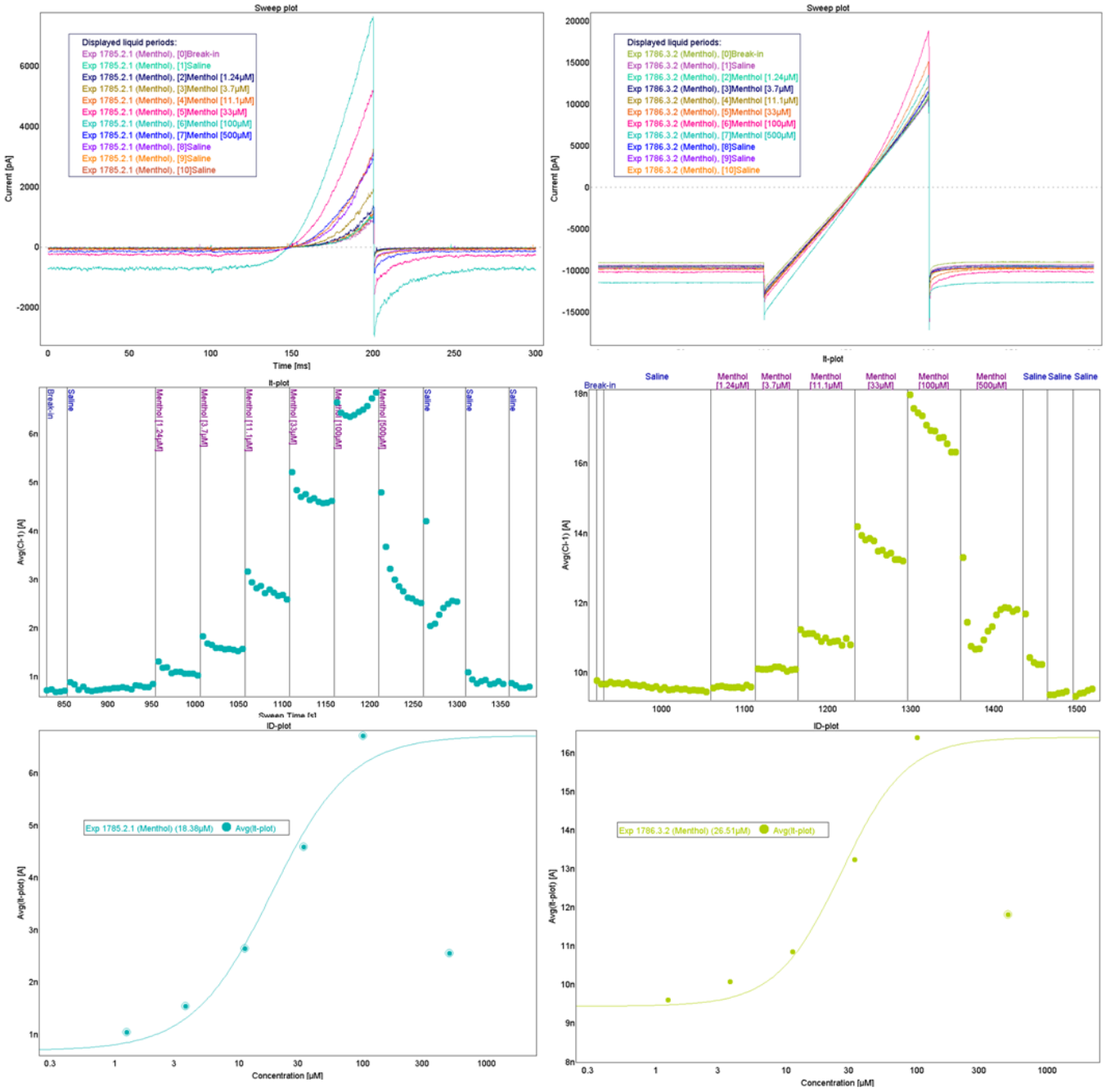

Figure 16 Left: Single-hole data in a dose-response experiment with -/-menthol. Right: multi-hole data in a dose-response experiment with -/-menthol. Top: raw data sweeps elicited with a ramp from -80 mV to +60 mV, middle: IT plot of current at +60 mV, bottom: Hill fit.

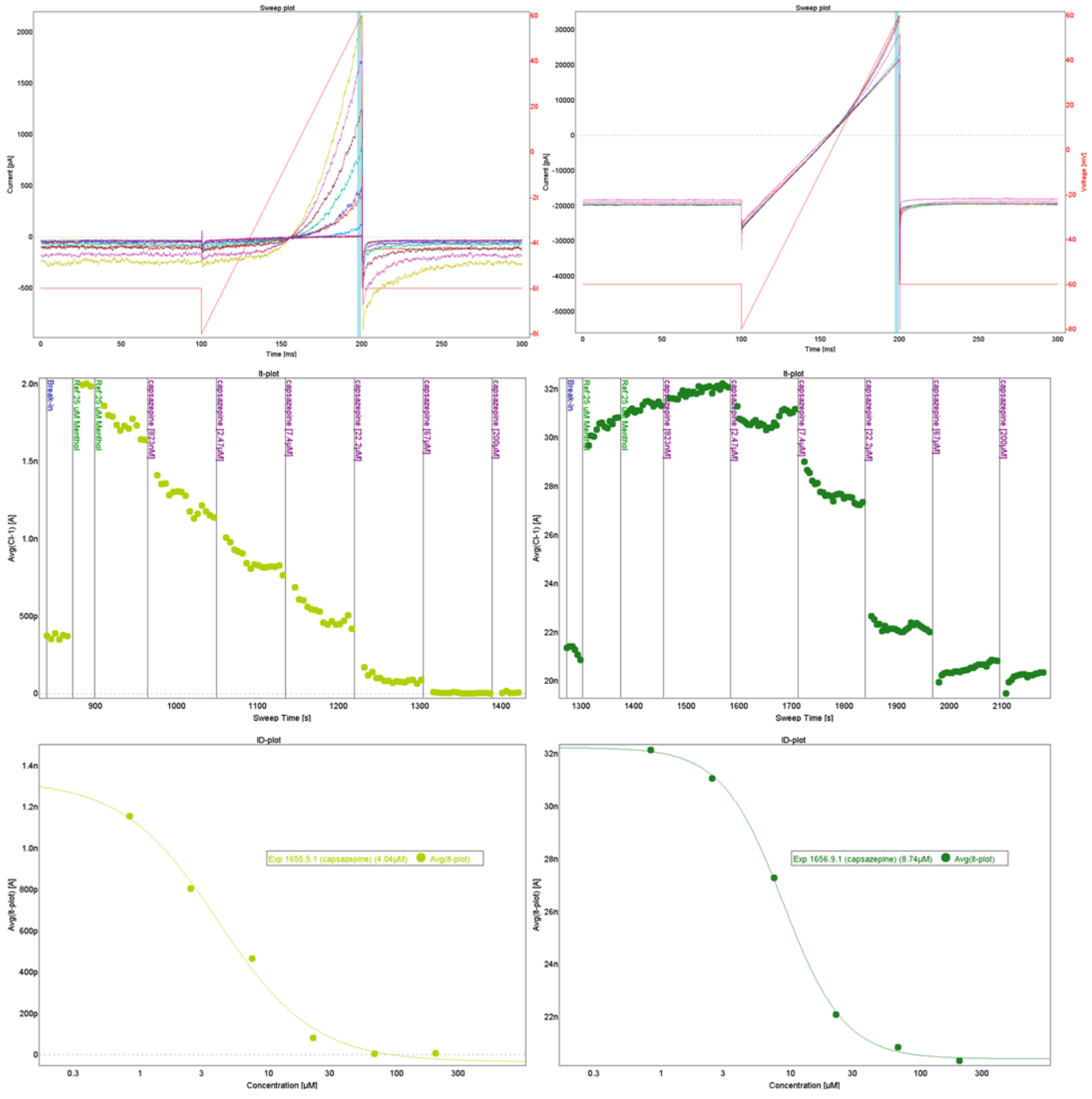

Figure 17 Dose-response experiment with capsazepine tested in the presence of 25 µM -/-menthol. Left: Single-hole data in a dose-response experiment with capsazepine. Right: multi-hole data in a dose-response experiment with capsazepine. Top: raw data sweeps elicited with a ramp from -80 mV to +60 mV, middle: IT plot of current at +60 mV, bottom: Hill fit.

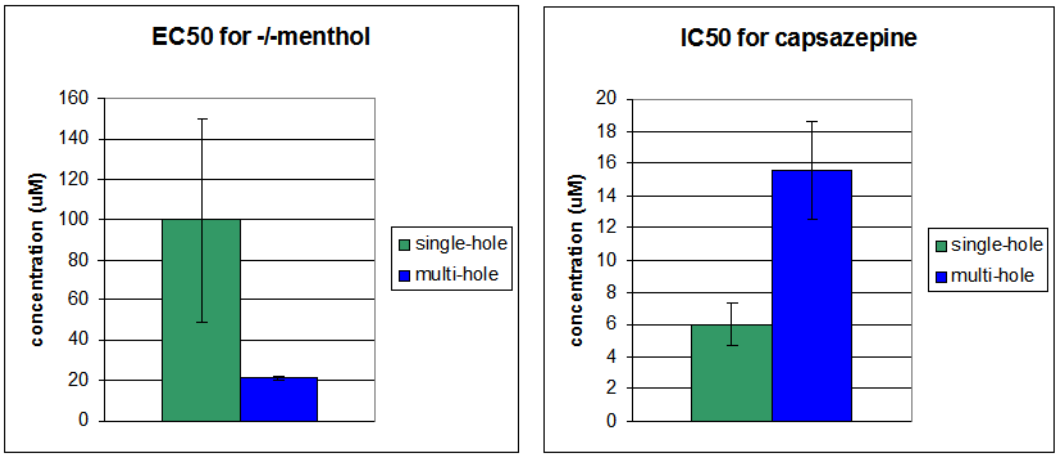

Figure 18 EC_{50} ± SD values obtained with –(-)-menthol and capsazepine in single-hole and multi-hole mode (n=3-10).

References

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Conclusion

CHO-TRPM8 and HEK-hTRPM8 cells perform well, and with high succes rates on QPatch. The QPatch can produce high-quality gigaseals and good recordings of the ion channel current. EC50 and IC50 estimations were successfully obtained on menthol and icilin and capsazepine, and were found to be within the range described in the literature. TRPM8 is also shown here to work well in the multi-hole mode on the QPatch X technology.