

Application Report:

CHO Ca_v1.2

QPatch

Validation on QPatch



Current from Ca_v1.2 expressed in CHO-K1 was tested on QPatch in both singlehole and multihole mode. Voltage dependence of activation and inactivation was evaluated and three different known compounds were tested to see if the pharmaceutical properties were as expected.

AR_PUBLIC16476-4

Introduction

L-type voltage gated calcium channels are involved in cardiac arrhythmia and hypertension and are therefore possible therapeutic targets. Current from Ca_v1.2 expressed in CHO-K1 was tested on QPatch in both single-hole and multi-hole mode. Success rates, IV characteristics, steady state inactivation and three different known compounds were tested to see if the biophysical and pharmaceutical properties were as expected. Furthermore it was tested how well the cells could be frozen down for "direct use" on the QPatch.

Materials & Methods

Cells

The cell line used was a CHO-K1 cell line expressing the human CACNA1C and CACNB2 and CACNA2D1 genes. CACNA1C encodes the pore-forming subunits of the L-type calcium channel subtype Ca_v1.2. CACNB2 and CACNA2D1 encode auxiliary subunits that modulate gating and pharmacological properties. The cell line was developed by Chantest and they were grown according to Chantest SOP.

Preparation and use of frozen cells for direct run were prepared as previously described in SOP.

Solutions

Intracellular (IC) (mM): 27 CsF, 112 CsCl, 8.2 EGTA, 10 HEPES, 4 Na-ATP, 2 NaCl, pH 7.2 (adjusted with CsOH), osmolarity app. 295 mOsm.

Extracellular (EC) (mM): 145 NaCl, 10 CaCl₂, 4 KCl, 10 HEPES, pH 7.4 (adjusted with NaOH).

On the day of experiment:

The intracellular solution is made up from two stock solutions on the day of experiment. The stock solutions are:

IC₁: 135 CsF, 10 HEPES, 10 NaCl, 1 EGTA, pH 7.2 (adjusted with CsOH).

IC₂: 140 CsCl, 10 EGTA, 10 HEPES, 5 ATP, pH 7.2 (adjusted with KOH).

IC₂ is made and stored free of ATP. ATP is first added to this internal and pH is adjusted with KOH to 7.2.

IC₁ and IC₂ are then mixed 20%/80% and pH and osmolarity is checked and adjusted if needed.

Osmolarty of EC is adjusted with glucose to app. 20 mosm more than IC.

Note: If linear rundown is present with the solutions above it is advised to change Na-ATP with Mg-ATP in the same concentration.

Protocols

The whole cell protocol used was standard single-hole CHO protocol and a standard CHO multi-hole protocol. The key parameters for the single-hole protocol are: positioning pressure -100 mBar for 30 sec. Rest period at -20 mbar and -90 mV for 3 minutes and whole cell formation by suction pulses starting at -250 mbar.

The multi-hole protocol is identical but with "timed" ticked on in the protocol. Due to this timing the wait period is 120 sec. longer.

Results

The overall success rate was the first parameter evaluated. It was found that the cells sealed up very well with the solutions described in materials and methods. A typical QPlate overview of a single-hole and a multi-hole plate is shown below in Figure 1. For single-hole it was found that seal resistance and whole cell resistance were $1350 \pm 178 \text{ M}\Omega$ and $650 \pm 72 \text{ M}\Omega$, respectively. The values for the same parameters in multi-hole were $36 \pm 2.6 \text{ M}\Omega$ for seal resistances and $40 \pm 3.3 \text{ M}\Omega$ for whole cells. These values are typical - also for other calcium cell lines that have been tested on the QPatch.

It should be noted that Ba²⁺ (44 or 5 mM) also was tested as the charge carrier and it was found that the seal resistances were reduced to about 30% of the what was seen with Ca²⁺, data not shown.

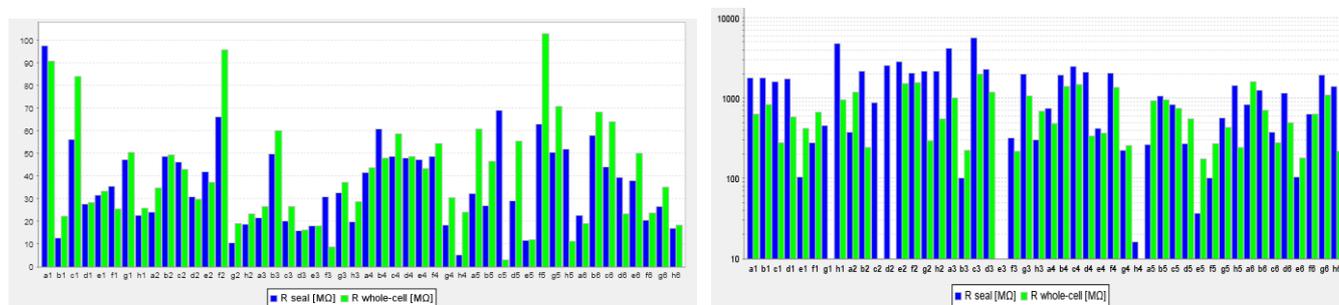


Figure 1. Seal resistances from a typical single hole (right) and multi hole (left) plate.

A breakdown of the performance of three plates is shown in Table 1.

| QPlate success rates | Single-hole | Multi-hole | Frozen cell run (single-hole) |
|--|-------------|------------|-------------------------------|
| No. of QPlates | 2 | 3 | 1 |
| Cell attachment (%) | 100 | 100 | 90 |
| Seal > 100 MΩ (%) | 37 | n/a | 54 |
| Seal > 1 GΩ (%) | 51 | n/a | 11 |
| Whole-cells (%) | 81 | 100 | 63 |
| Completed experiments (%) | 81 | 100 | 36 |
| Representative whole-cell lifetime (min) | 30 | 20 | 20 |

Table 1. Overview of Ca_v1.2 success rates in different experiment phases.

This cell line is tetracycline inducible and it was found that the time of induction/cell confluence when inducing were the important parameters. Induction was tested at different confluences and it was found that the optimal confluence was about 80% for best patch and expression performance.

On the QPatch a start cell density of 2-4 mill cells/mL is needed for optimal seal performance. Since tetracycline inhibits cell growth the problem usually seen when induction was done too early was that the cell yield was too low resulting in lower than optimal patch performance on the cell line.

However, growing the cells too confluent in the mother flask (>80%) was also found to reduce the expression of the ion channel to virtually zero. This loss of expression is non-reversible and the only thing to do is to take up a new batch of cells. This is a common issue when CHO cells are used as the host cell and just standard awareness of this behavior should be enough to ensure this from happening.

The expression level found in QPatch experiments is shown as histograms for single-hole and multi-hole in Figure 2. It was found that 67% of the whole cells obtained expressed more than 100 pA Ca_v1.2 current in single-hole mode. This was increased to 95% of sites with more than 100 pA Ca_v1.2 current by using multi hole plates. Mean current for the cells that expressed more than 100 pA was found to be -1.3 ± 0.1 nA for single-hole and -3.5 ± 0.3 nA for multi-hole.

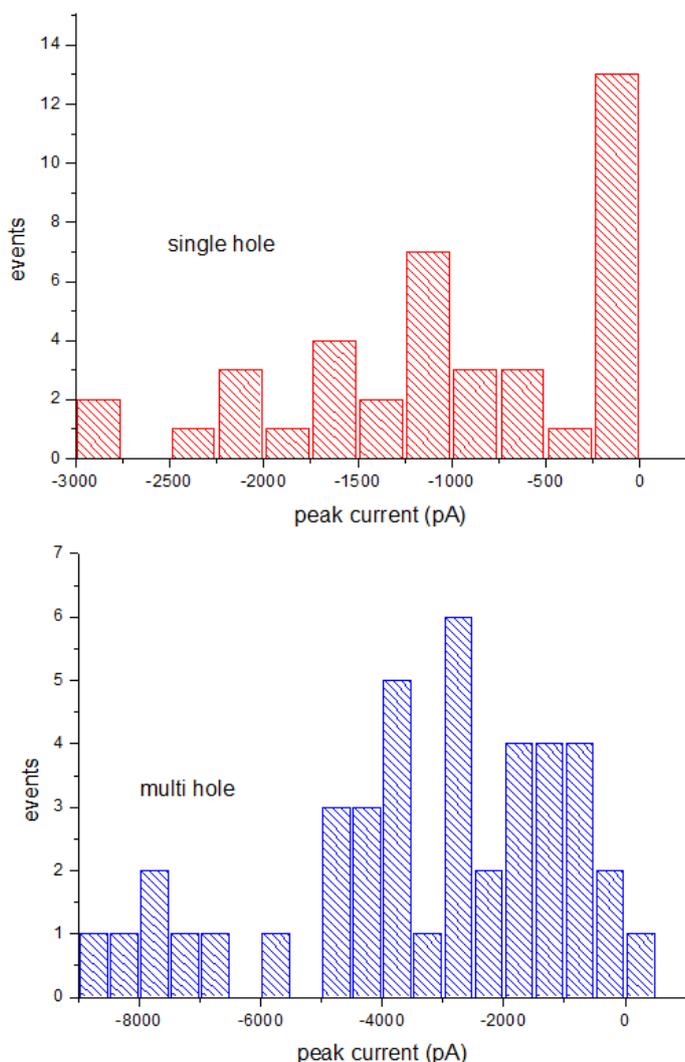


Figure 2. Comparison between single and multi-hole Ca_v1.2 peak current distribution.

It can be convenient to have cells frozen down that are ready to use on the QPatch especially inducible CHO cells since the induction time (2 days) narrows the work day window where the cells are usable. It was therefore tested how well induced CHO Ca_v1.2 cells performed in a "ready to run" experiment. One T175 flask gave a yield of 4 x 1.5 mL vials of cells with 2 mill cells/mL. One vial of cells is enough for one run using the "no QStirrer" option on the QPatch.

It was found that the sealing resistances for frozen cells were 17 ± 2 Mohm and the current amplitudes were 3.9 ± 6 nA for multi-hole mode. For single-hole the same parameters were found to be 1.2 ± 0.2 Gohm and -1.6 ± 0.3 nA. It was therefore concluded that these cells were well suited for freezing down and running them directly on the QPatch.

IV characteristics and steady state inactivation

Ca_v1.2 is, as the name implies, is voltage gated and the voltage dependence was therefore investigated by applying different sets of voltage step protocols. It was found that with the solutions used here maximum current was seen by stepping to 20 mV. Figure 3 shows typical sweeps from an IV experiment. Figure 4 shows a typical IV-curve derived from such a voltage protocol.

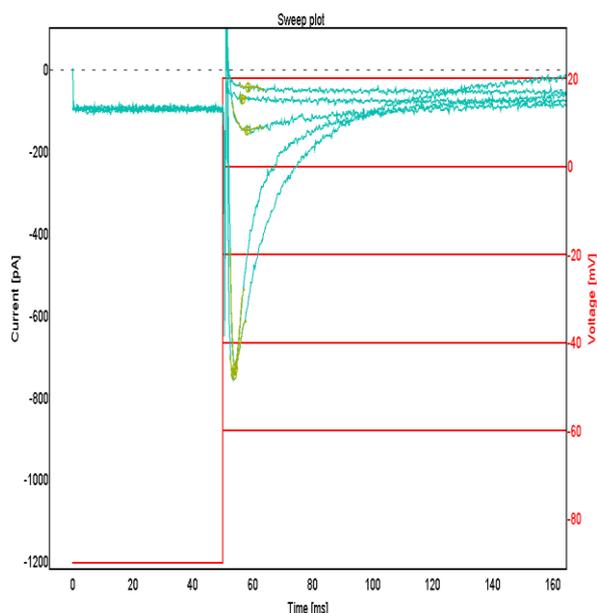


Figure 3. Typical Cav1.2 traces induced by stepping to increasingly depolarized potentials. For clarity only some of the traces are shown.

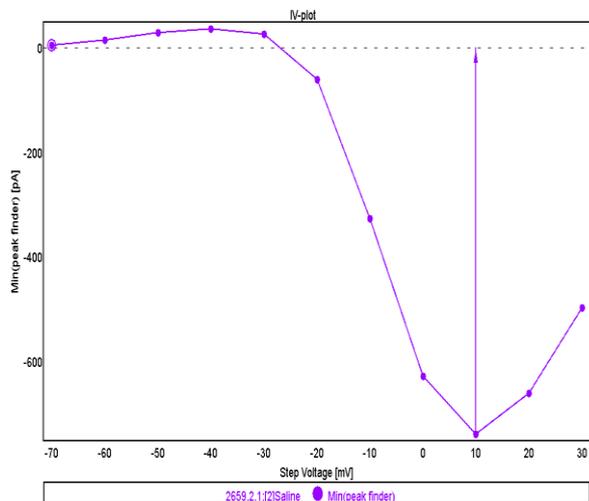


Figure 4. Ca_v1.2 current response as a function of step potential. Maximum current was found to be induced by depolarizing to 10 mV. This was in the following experiments the potential used where only one step potential was used.

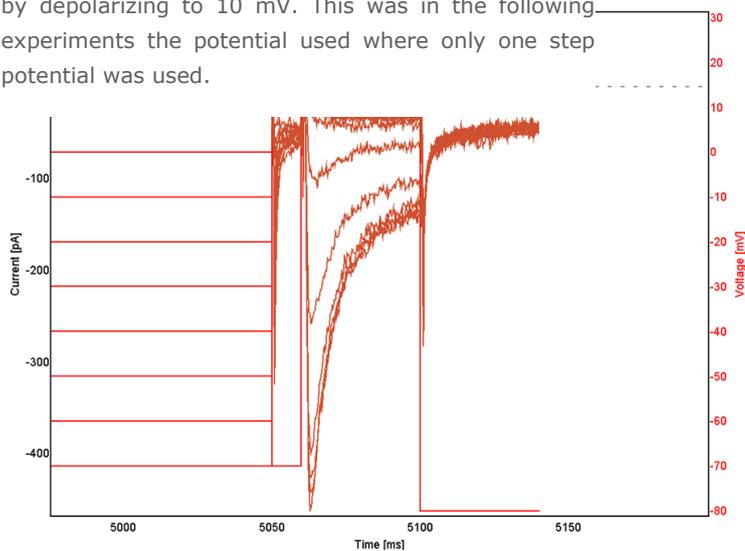


Figure 5. Calcium currents elicited at +10 following a 5 sec. prepulse at potentials from -70 to +30 mV.

$V_{1/2}$ for inactivation was determined to -30 ± 2 mV by stepping to increasingly depolarizing potentials for 5 seconds followed by a 10 ms step to -90 mV and then stepping to $+10$ mV where the response was measured. Figure 5 shows the resulting sweeps from this protocol and Figure 6 shows the derived Boltzmann curve from which $V_{1/2}$ was determined. These characteristics are in good agreement with published values. (Catterall et. al 2005.)

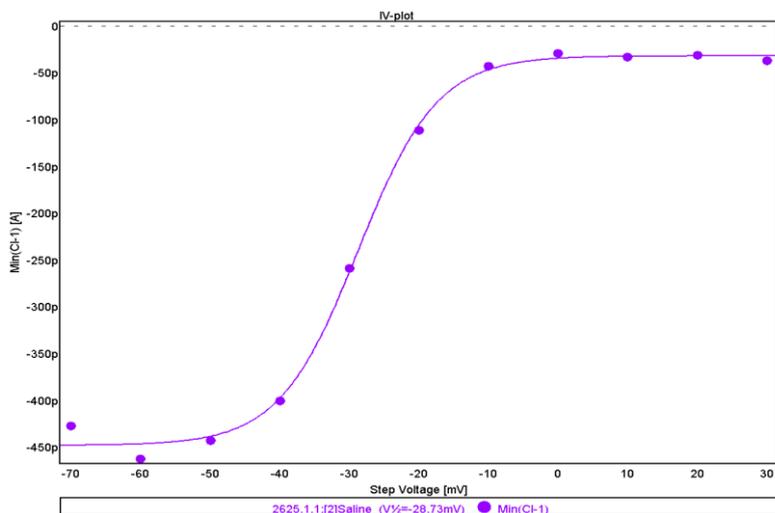


Figure 6. Inactivation curve fitted to the Boltzmann equation. $V_{1/2}$ was found to be -30 ± 2 mV.

Signal stability

Voltage gated calcium channels tend to be problematic due to instability of the current. It was found that with the solutions specified in materials and methods the current response using a 100 ms step protocol to +10 mV was stable over prolonged periods of time as exemplified in Figure 7.

In some cases it has been found that the current can run down linearly with Na-ATP. If that is the case it is recommended to use 4 mM of Mg-ATP.

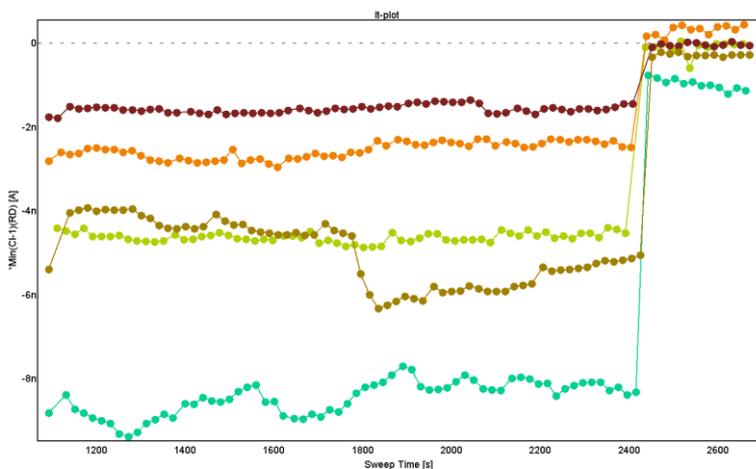


Figure 7. IT-plot from 5 different sites in a multi-hole dose response experiment where only EC is added. A saturating concentration of Nifedipine ($10 \mu\text{M}$) is added in the end of the experiment.

Pharmacology

Three different test compounds, nifedipine, verapamil and cisapride were tested on the channel to test how known pharmacology worked.

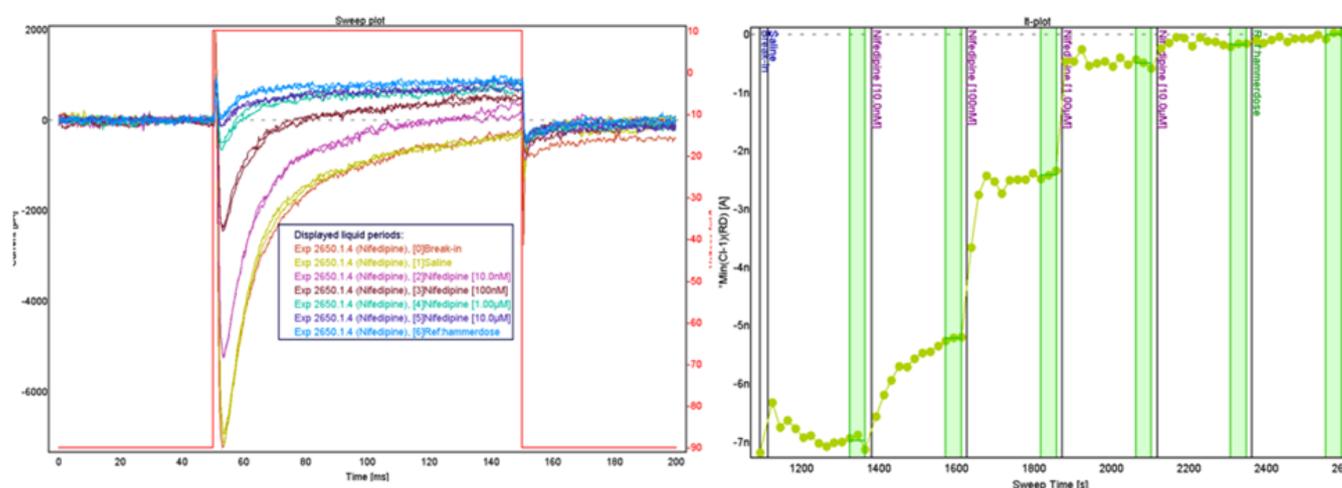


Figure 8. Raw data and IT-plot from a typical nifedipine experiment. The cell is exposed to four increasing concentrations of nifedipine followed by a saturating concentration of blocker to determine the amount of background current. It can be seen that the current starts off fairly stable and reaches steady state fast at each concentration step. This was found to be very typical of nifedipine action.

The experiments were set up as 4 point dose response experiments were 4 increasing concentrations of compound were added to each cell followed by a saturating concentration of nifedipine (10 μM) to block all Ca_v1.2 current. Each concentration was on for app. 5 minutes before next concentration was added.

The concentrations used were made up as 10 fold dilutions with 3 and 10 μM as the highest concentration in the case of nifedipine, 30 and 100 μM for cisapride, 300 and 100 μM for verapamil. Ca_v1.2 tonic response was elicited by a depolarizing test pulse to 10 mV @ 0.05 Hz.

Typical sweeps and an IT-plot from a nifedipine experiment are shown in Figure 8.

| IC ₅₀ for reference blockers of Ca _v 1.2 (μM) | | |
|---|-------------|------------|
| | Single-hole | Multi-hole |
| Verapamil | 44 | 71 |
| Cisapride | 12 | 31 |
| Nifedipine | 0.024 | 0.052 |

Table 2. IC₅₀ values of reference compounds on Ca_v1.2 determined on single and multi hole

This approach allows for a combined 8 point dose response curve for each compound. The experiment combination is handled within the QPatch assay software. The experiments were done both on single-hole and multi-hole plates. The obtained IC₅₀s can be seen in Table 2.

By normalizing the Ca_v1.2 current and combining the experiments as described above it is possible to make a grouped plot with data from all the experiments that can be fitted to the Hill equation to give a combined IC₅₀ value. See Figure 9 for an example of such a combined curve.

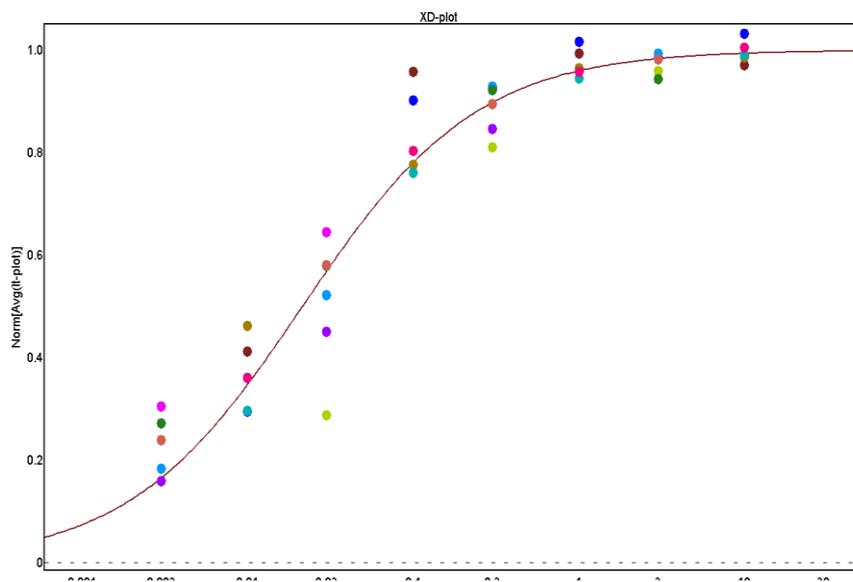


Figure 9. Normalized responses from 10 multi-hole sites as a function of nifedipine concentration. Each site was either exposed to 0.003-30 μM or 0.001-10 μM. IC₅₀ was determined by fitting the points to the Hill equation. Each dot color represents data from one experiment.

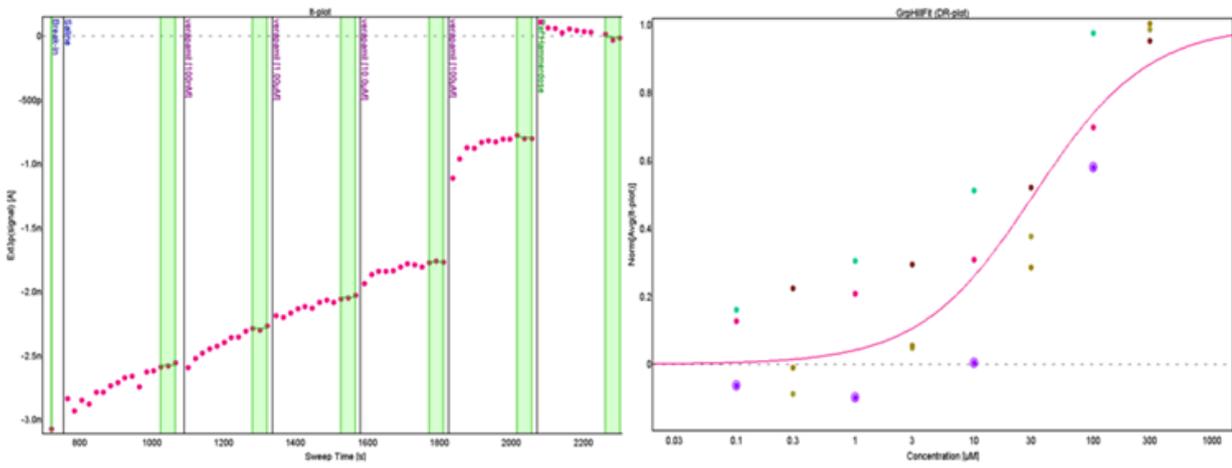


Figure 11. IT-plot and derived dose response plot combined from 8 individual single-hole experiments.

Typical cisapride IT-plot and dose response plots are shown in Figure 10 and similar data from the verapamil experiments are shown in Figure 11. These values are found to be well within previously reported values for these compounds (Renaud et al. 1986; Janis et al. 1983). These experiments verify that the cell line can be used for profiling studies on the QPatch.

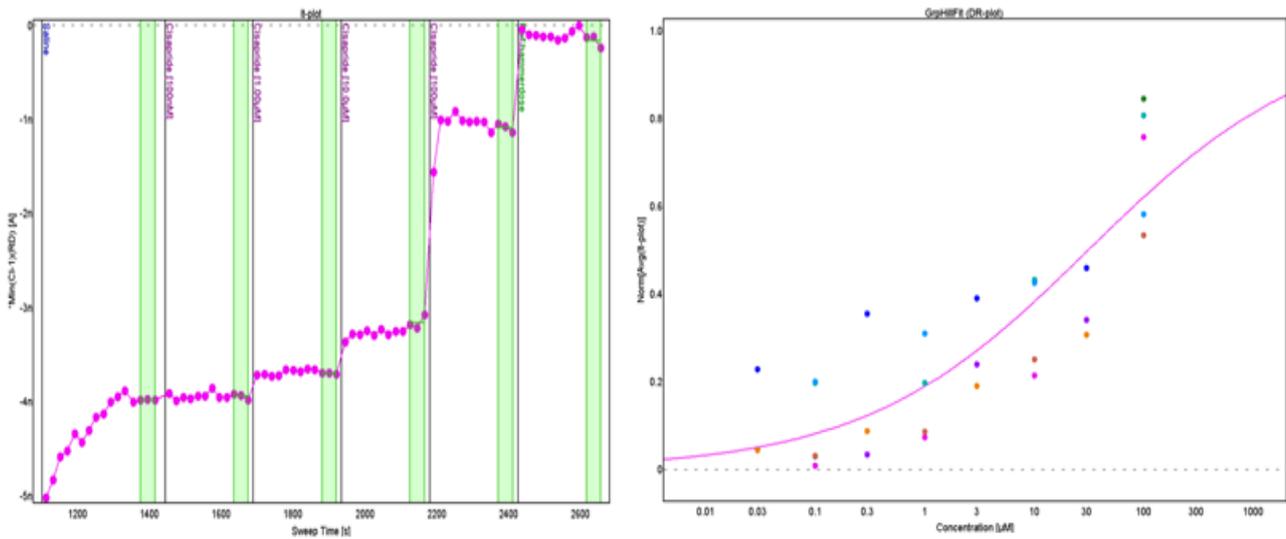


Figure 10. Typical cisapride IT-plot and derived dose response data from 6 individual experiments in multi-hole mode

References

Renaud JF, Schmid A: J Physiol (Paris). 1986;81(3): 177-84

Janis RA, Scriabine A: Biochem Pharmacol. 1983; 32(23): 3499-507

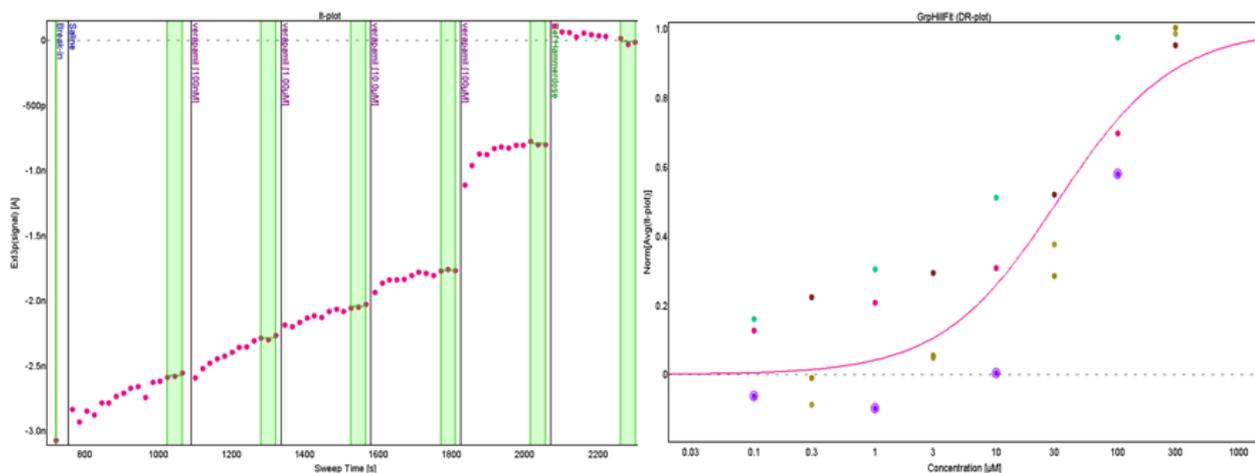


Figure 11. IT-plot and derived dose response plot combined from 8 individual single-hole experiments.

Conclusion

CHO Ca_v1.2 has been tested on QPatch and the cell line was found to be a well suited for the QPatch. We have found that IV characteristics and pharmacology to within expected ranges and we therefore conclude that this Ca_v1.2 on QPatch can be used for profiling purposes.

The cell line was found to be very useful especially in multi-hole mode due to the fact that the cell line patches very well on the QPatch and multi-hole mode increases the number of functional sites by 50%.