

Application Report:

CHO TREx-P2X₃

QPatch

P2X₃ tested on the QPatch



This report contains data and assay development tips and tricks for QPatch experiments on the P2X₃ receptor.

AR_PUBLIC14580-5

Introduction

In this report the applications of the CHO TREx-P2X₃ cell line (hereafter called CHO P2X₃) are described. The purpose was to determine the EC₅₀ value for $\alpha\beta$ -methyl ATP, and the IC₅₀ value of PPADS on the P2X₃ receptors. All data presented was obtained with QPatch, and analysed with the QPatch assay software.

Materials & Methods

Hexokinase

Hexokinase catalyzes the phosphorylation of D-hexose sugars at the C6 position utilizing ATP as a phosphate source. Thus, the aim of having hexokinase in the saline buffer is to reduce the amount of ATP leaked from cells in order not to have this natural agonist in the cell suspension.

D-Glucose + ATP Hexokinase > D-Glucose 6-Phosphate + ADP

Hexokinase requires Mg²⁺ for its activity (K_M = 2.6 mM) for activity; the EC ringer solution contains MgCl₂ (1 mM), and glucose (10 mM) is added to the ringer solution.

Unit definition: One unit will phosphorylate 1.0 μ mol of D-glucose per min at pH 7.6 at 25 °C. We use 30 U/ml (previously optimized by Sophion).

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 a final cell density was approximately 3-5 mio. cells/ml.

Ringer's solutions

IC 0.0.0, pH 7.2 (in mM): 5.374 CaCl₂, 1.75 MgCl₂, 10 HEPES, 120 KCl, 31.25/10 KOH/EGTA, 4 Na₂ATP.

EC (Reference) pH 7.4 (in mM): 150 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES 10 Glucose*, 30 U/ml Hexokinase**

EC (cell preparation) pH 7.4 (in mM): 150 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 Glucose*

* necessary when hexokinase is used

** the best results were obtained when the hexokinase was used after whole-cell establishment. Therefore two EC ringers were prepared. The ringer used for cell preparation contained glucose, but not hexokinase, as the cells sealed better in a ringer without hexokinase. The "reference ringer", which was used for wash periods contained glucose and hexokinase.

Results

Determination of minimum cycle duration and number of washes

Initial experiments were done to determine the agonist application interval and number of washes to obtain reproducible responses after agonist application ($\alpha\beta$ -methyl ATP 1 μ M). It was observed that recovery from desensitization was obtained using 6 washes in combination with 360 s agonist application interval. (The latter is in QPatch Assay Software terms denoted as minimum cycle duration). Typical current sweeps are shown in Figure 1.

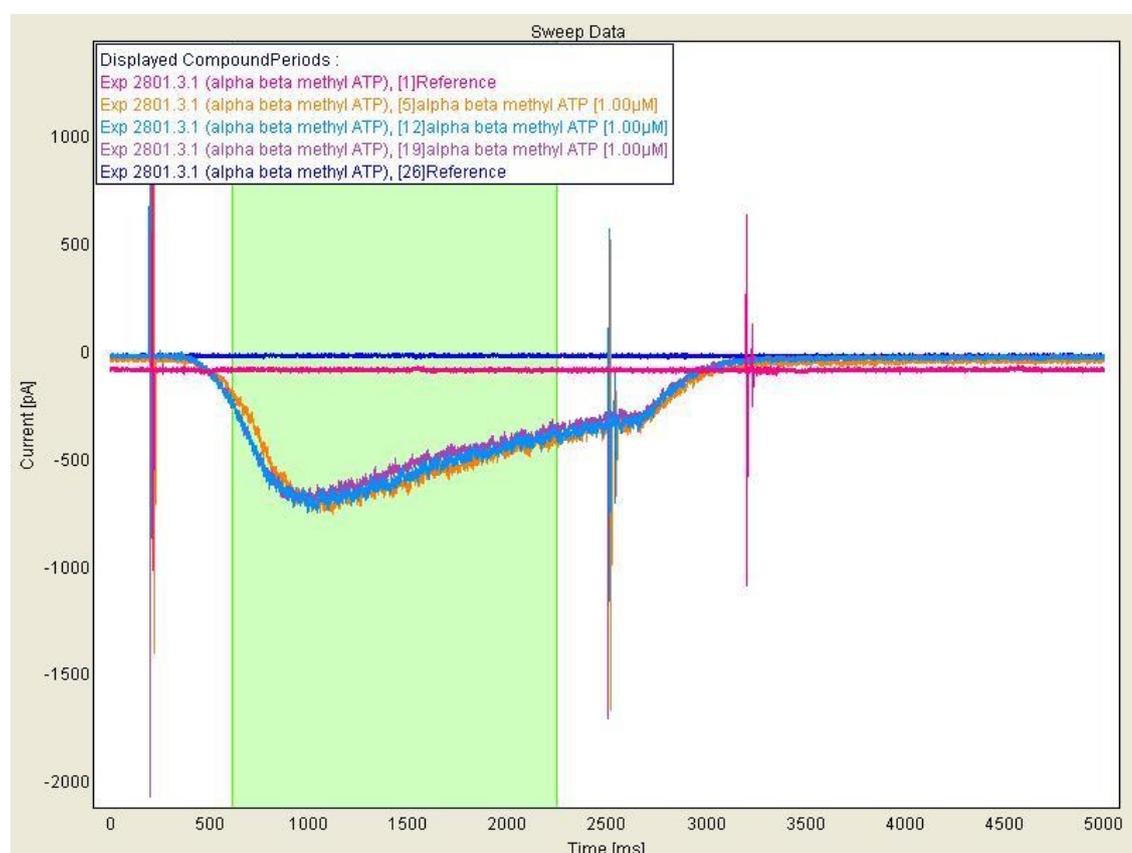


Figure 1. Recovery from desensitization of P2X₃ receptors from the agonist $\alpha\beta$ -methyl ATP (1 μ M applied three times) obtained with cycle duration of 360 s and 6 s wash periods. The green bar represents the position of the user defined cursor for current amplitude measurements.

The peak current was determined for each liquid application from the user defined cursor and the resulting IT plot is shown in Figure 2.

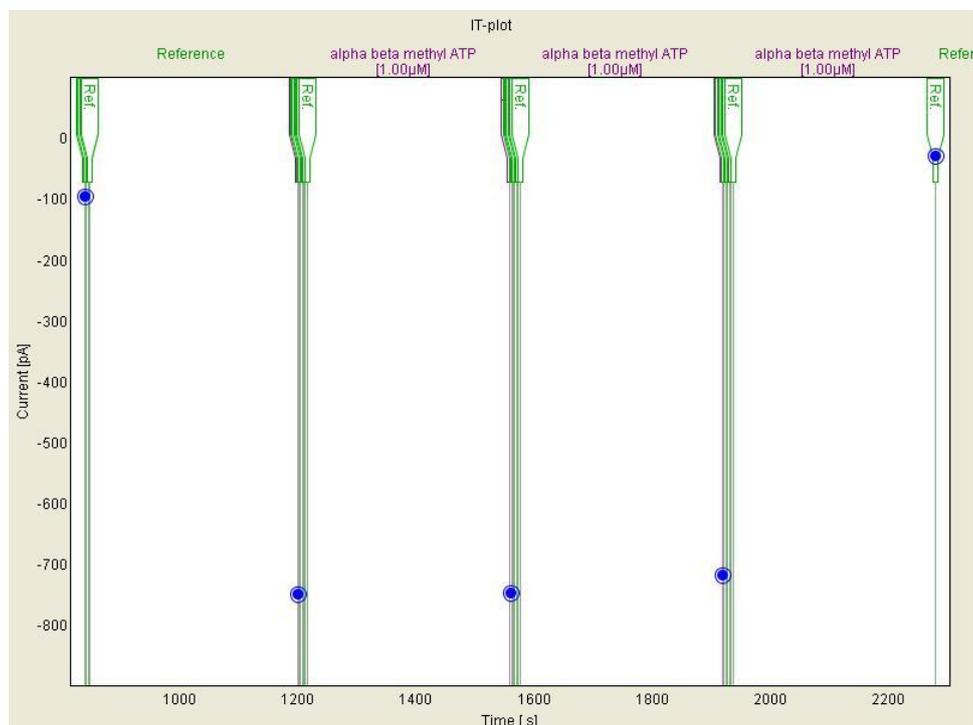


Figure 2. IT plot from experiment with three repetitive agonist ($\alpha\beta$ -methyl ATP 1 μM) applications with minimum cycle duration of 360 s and 6 washes after each application. Note that throughout the experiment an EC ringer with glucose and hexokinase was used (see materials and methods).

EC₅₀ determination of $\alpha\beta$ -methyl ATP for P2X₃ receptors

$\alpha\beta$ -methyl ATP dose-response experiments were done with a minimum cycle duration of 360 s and 6 washes after each application. Currents from a typical dose-response experiment are shown in Figure 3.

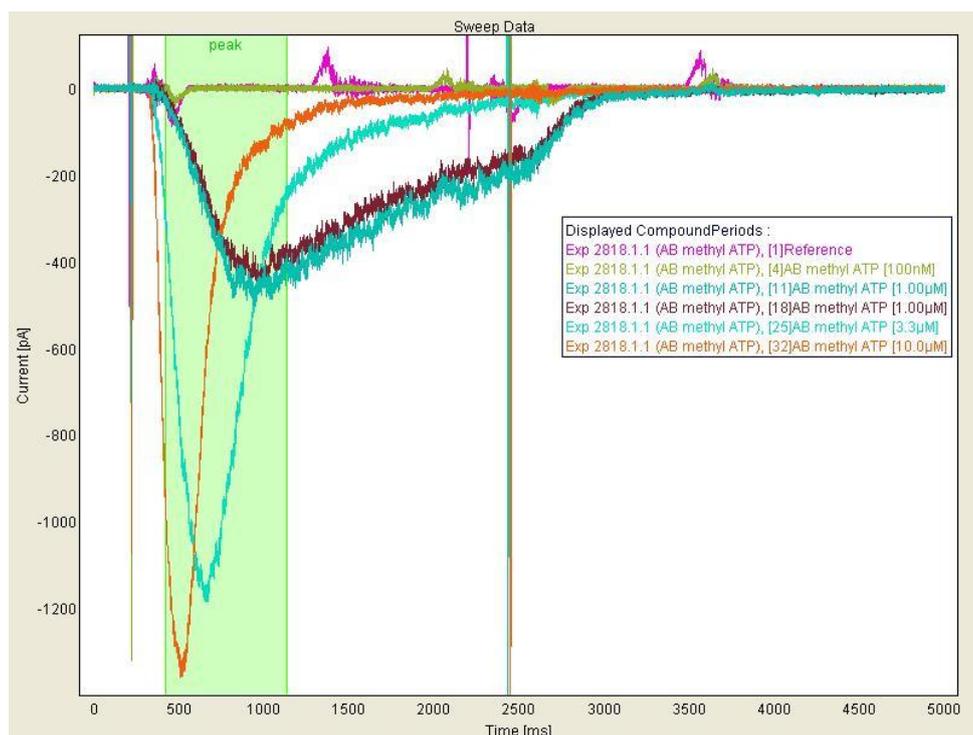


Figure 3. P2X₃ current sweeps from $\alpha\beta$ -methyl ATP dose-response experiment. The green bar represents the position of the user defined cursor for current amplitude measurements.

The corresponding Hill fit for the agonist is shown in Figure 4, where the EC₅₀ value was determined to 1.4 μM (mean 1.2 μM ± 0.3, n=27).

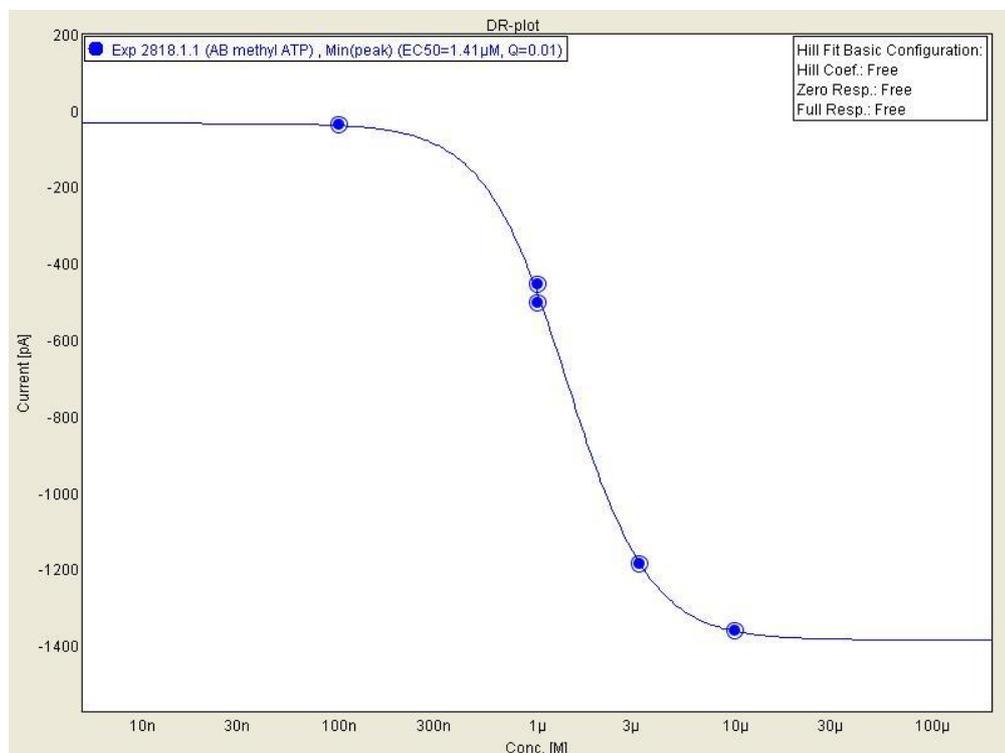


Figure 4. Hill fit and EC₅₀ determination of αβ-methyl ATP for P2X₃ receptors. 1 μM agonist was added twice to show the reproducibility of the data.

IC₅₀ determination of PPADS for P2X₃ receptors

For IC₅₀ determination of the antagonist PPADS for P2X₃ receptors, experiments were done with a minimum time between applications of 360 s and 6 washes after each application. The PPADS compounds at concentrations of 0.1, 1, 3, 10 and 30 μM were applied sequentially using αβ-methyl ATP (3.3 μM) as the agonist. PPADS was preincubated for 360s prior to adding the PPADS and the αβ-methyl ATP agonist. Typical P2X₃ currents from a dose-response experiment are shown in Figure 5.

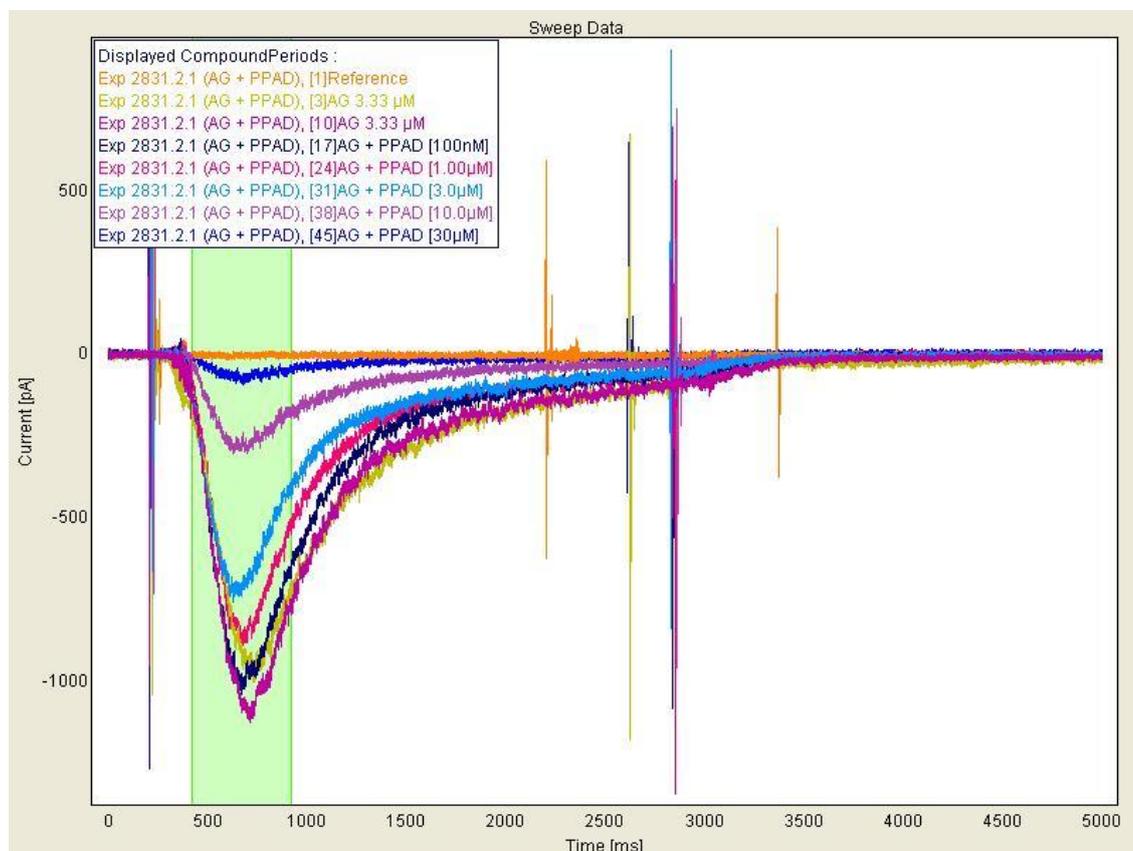


Figure 5. P2X₃ current sweeps from PPADS dose-response experiment. In this experiment the agonist (α -methyl ATP) concentration was 3.3 μ M. The green bar represents the position of the user defined cursor for current amplitude measurements.

The corresponding Hill fit for the antagonist is shown in Figure 6, where the IC₅₀ value was determined to 5.7 μ M (mean 5.5 μ M \pm 1.8, n=17 @ 3.3 μ M α -methyl ATP).

Similar results were determined using the agonist, α -methyl ATP at a concentration of 1 μ M. In these experiments the IC₅₀ value for PPADS was determined to 6.0 μ M \pm 1.8, n=19. There was no significant difference regarding the IC₅₀ value obtained for PPADS with respect to the concentration of the agonist – Figure 7. This confirms that PPADS does not act as a competitive inhibitor as it is also described in the literature (Li 2000).

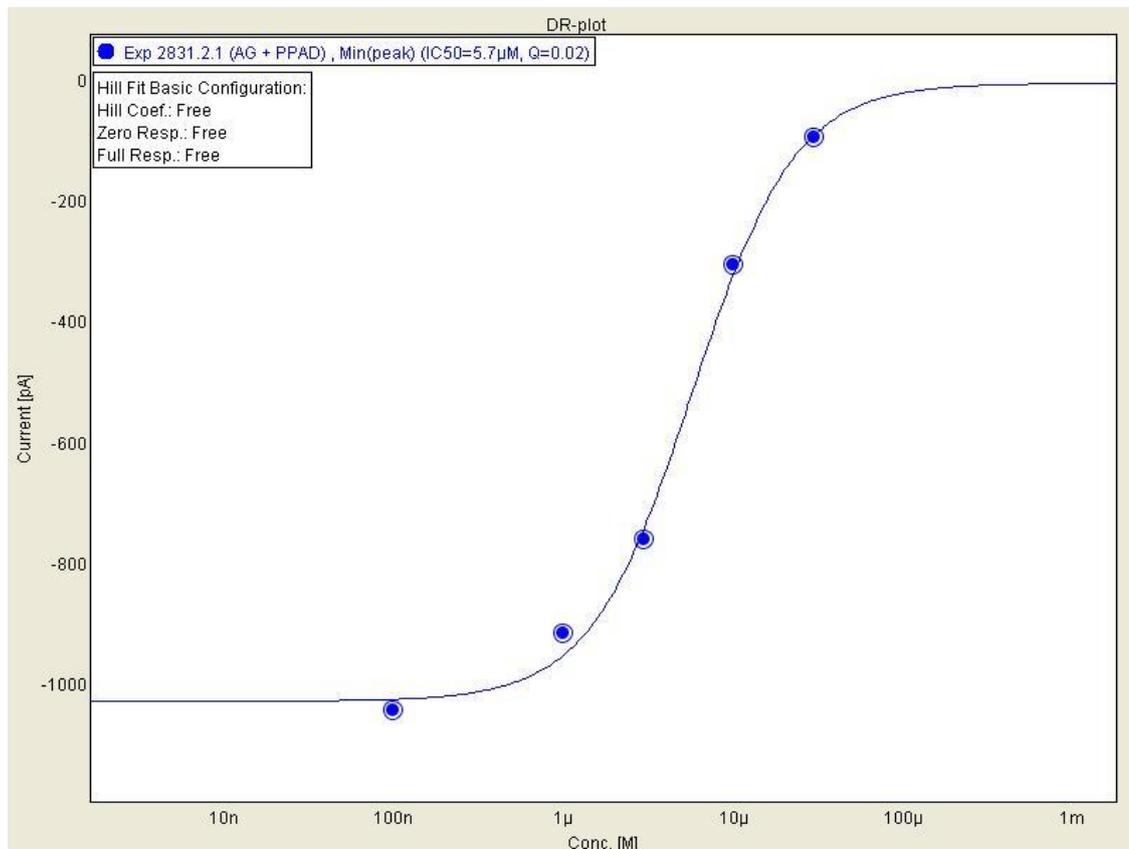


Figure 6. Hill fit and IC₅₀ determination PPADS for P2X3 receptors

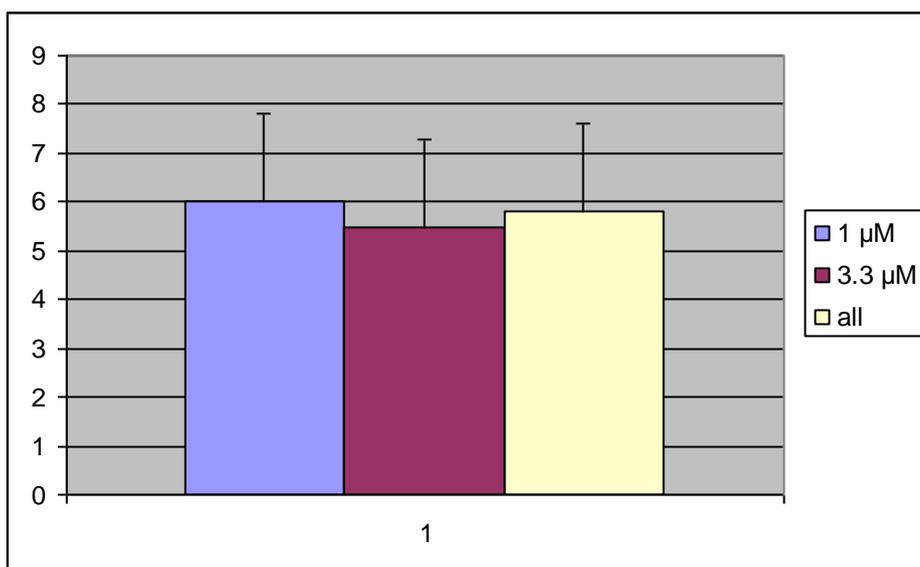


Figure 7. Summary of the IC₅₀ values for PPADS on P2X3 receptors obtained with different agonist, qβ-methyl ATP, concentrations. The yellow column represents the cumulated data. The mean IC₅₀ value for the summarized data was 5.8 µM ± 1.8, n=36, obtained using 4 QPlates.

EC₅₀ determination of CTP for P2X₃ receptors

CTP dose-response experiments were done with a minimum cycle duration of 180 s and 6 washes after each application. Currents from a typical dose-response experiment are shown in Figure 8.

The corresponding Hill fit for the agonist is shown in Figure 9, where the IC₅₀ value was determined to 13.0 μ M (mean 11.1 μ M \pm 3.6, n=9).

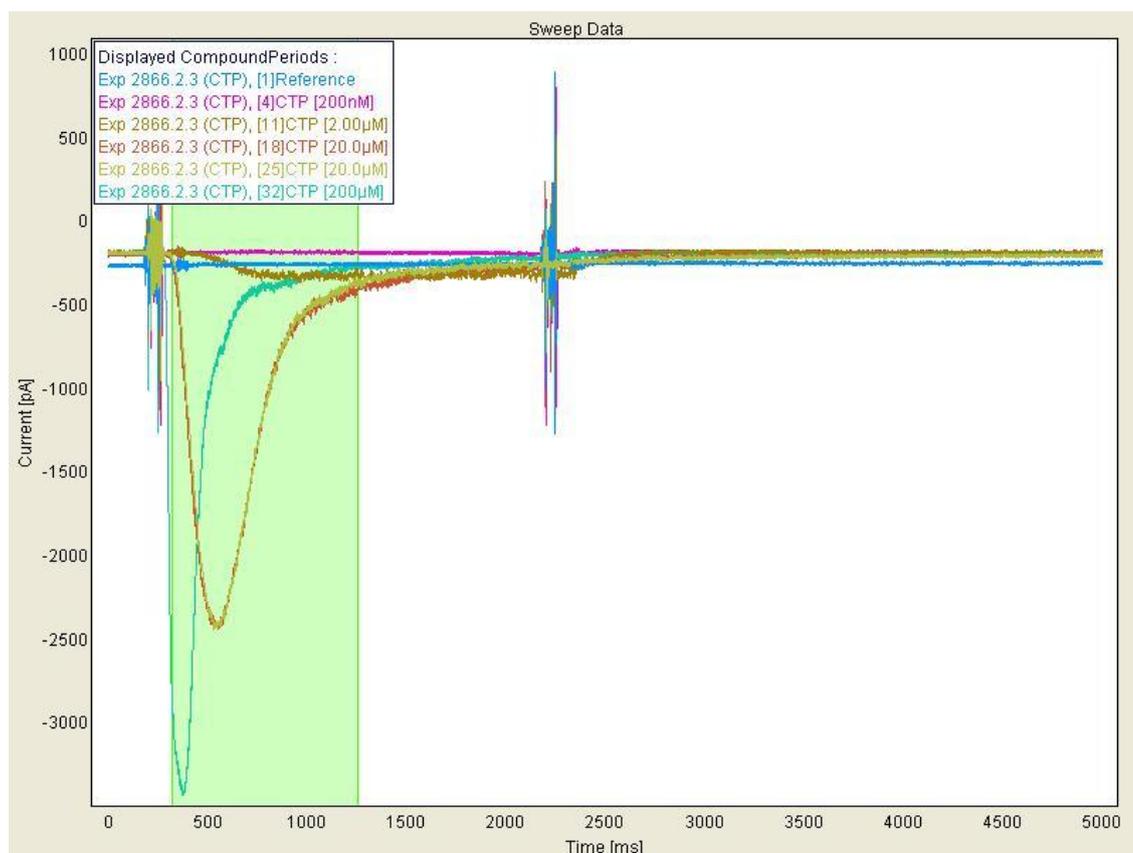


Figure 8. P2X₃ current sweeps from CTP dose-response experiment. The green bar represents the position of the user defined cursor for current amplitude measurements.

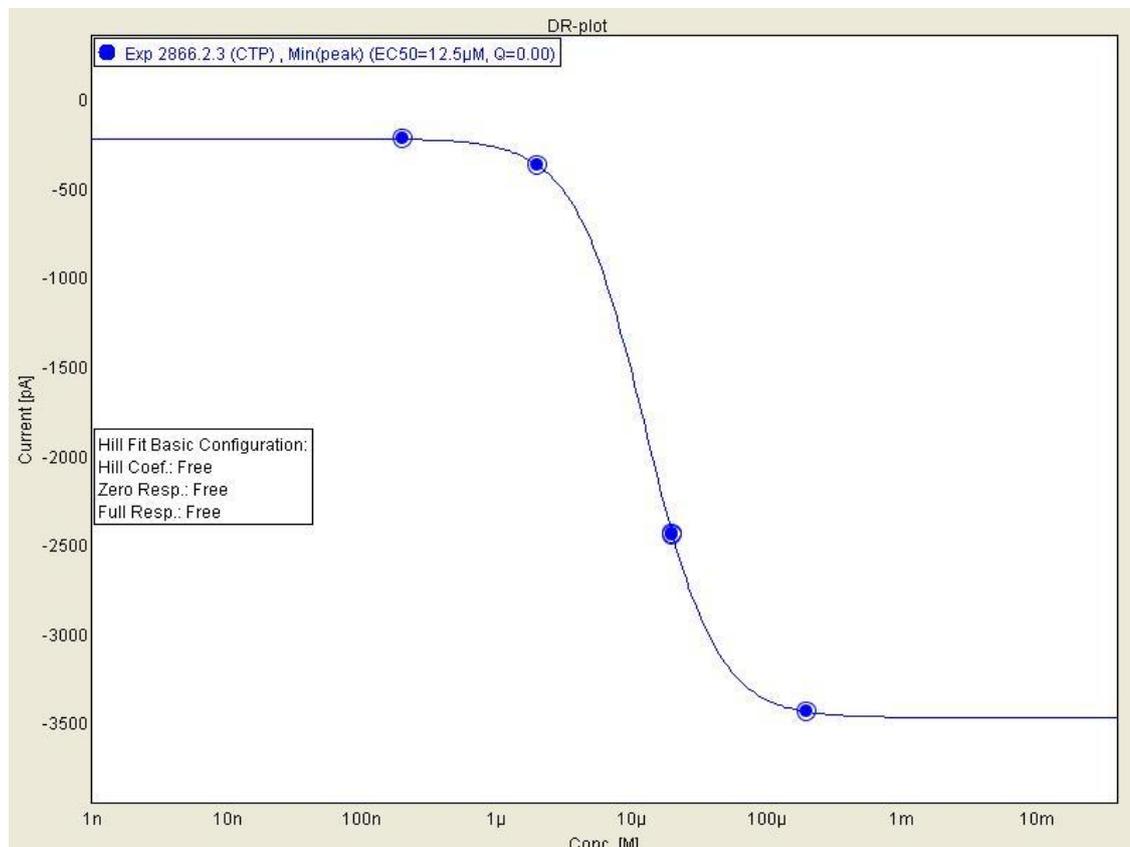


Figure 9. Hill fit and EC₅₀ determination CTP for P2X₃ receptors.

Summary of the results

Table 1 below summarises the results obtained on the P2X₃ receptors.

	EC ₅₀ (µM)	IC ₅₀ (µM)	Literature values (µM)
αβ-methyl ATP	1.2 ± 0.3		2.4
CTP	11.1 ± 3.6		17.3
PPADS		5.8 ± 1.8	2.5

Table 1. EC₅₀ and IC₅₀ obtained on P2X₃ receptors.

QPlate overview

It was observed that high seal resistances in the giga seal mode was easily obtained with CHO P2X₃ cells on QPatch, and the membrane resistance was tight also after the whole cell configuration was established. The experiments lasted typically 45 minutes during which the currents were recorded with minimal leak development. Figure 10 shows QPlate statistics from a typical experiment.

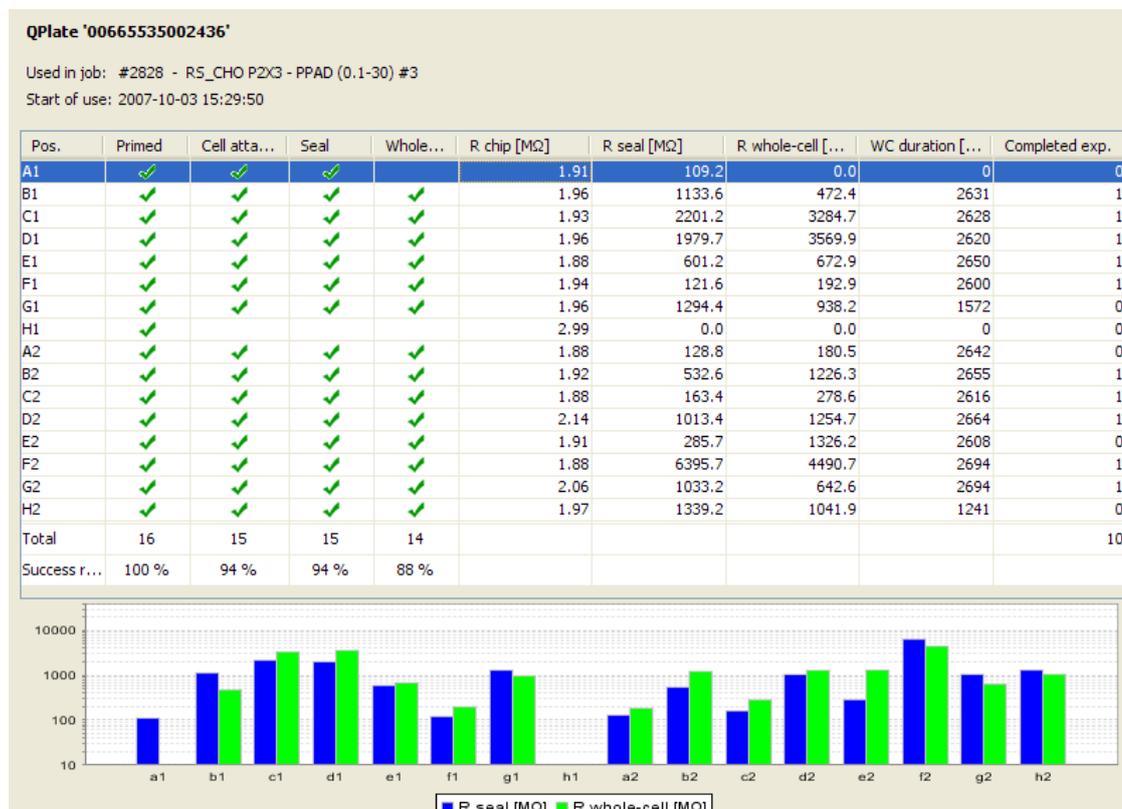


Figure 10. QPlate statistics from a typical experiment with CHO P2X₃ cells. The table lists success or failure for each QPlate position (A1 to H2), priming of the measurement site, cell attaching, formation of seal ($R_{\text{seal}} > \text{seal criterion}$), and formation of whole-cell configurations. Overall success rates are listed in the lower line of the table. In addition the measured electrical resistance at each measurement site (R_{chip}), the cell-attached configuration resistance (R_{seal}), the whole-cell resistance ($R_{\text{whole-cell}}$), the whole-cell duration, and the number of completed experiments are listed.

References

- Pratt et al. 2005 Use-dependent inhibition of P2X₃ receptors by nanomolar agonist. *J Neurosci* 25(32); 7359-7365
- Li 2000 Novel mechanism of inhibition by the P2 receptor antagonist PPADS of ATP-activated current in dorsal root ganglion neurons. *J Neurophysiol* 86(5); 2533-41

Conclusion

The CHO-P2X₃ cells sealed well on the QPatch; giga seals and the whole cell configuration were easily obtained, and the average number of completed dose-response experiments pr. QPlate was 10.5 ± 2.9 , n=11 QPlates

The EC₅₀ values determined by the QPatch assay software were close to literature values (for Alpha-beta methyl ATP and CTP).

The IC₅₀ value for PPADS was within the expected value.