SOPHION BIOSCIENCE

Application Report

Stable recordings of $P2X_{2/3}$ pharmacology on Qube 384

Short and precise ligand-exposure times and thorough wash prevents rundown and ensures high stability in ligand-gated ion channel assays on Qube.

Summary

- Assay with high stability, high success rates (93%) and low run down.
- Liquid stacking in the pipettes enables short and flexible ligand exposure times (down to 0.8 s).
- The microfluidic channels of the QChip ensure rapid and complete solution exchange, which together with liquid stacking results in a very stable assay without the use of hexokinase.
- The high stability enables cumulative antagonist dose-response experiments.

Introduction

The ATP-gated $P2X_2$ and $P2X_3$ receptors are cation-permeable ligand-gated ion channels that open in response to extracellular adenosine 5'-triphosphate (ATP) binding. They are widely expressed in the body, including the peripheral nervous system, making them an interesting target against pain[1–3].

When $P2X_2$ and $P2X_3$ receptors are expressed together, the functional receptors will be trimeric[3] and function either as $P2X_{2/3}$ heteromers, $P2X_2$ homomers, or $P2X_3$ homomers. The ratio between $P2X_2$ and $P2X_3$ homomers and $P2X_{2/3}$ heteromers varies from cell to cell, so to limit the variation between sites, only multihole QChips with ten patch holes per site are used in the assays.

$P2X_2$ and $P2X_3$ receptors display fast and extensive desensitization, making assays vulnerable to rundown due to poor compound washout or insufficient recovery. Here we show an assay with high stability, high success rates, and low rundown.

Results and discussion

Evaluation of receptor kinetics

$P2X_2$ homomers are insensitive to αβ-methylene ATP (αβmeATP)[1] and CTP[4] but sensitive to ATP, whereas both $P2X_3$ homomers and $P2X_{2/3}$ heteromers respond to all three agonists[5,6]. However, both activation and desensitization kinetics differ, with $P2X_3$ homomeric receptors generally having fast desensitization, especially for αβmeATP[1]. As shown in Figure 1A, the responses clearly vary depending on the agonist, showing that both $P2X_2$ and $P2X_3$ homomers and $P2X_{2/3}$ heteromers are expressed in the cells.

To enable short ligand exposure time liquids can be stacked in the pipettes so the exposure time can be precisely customized (down to 0.8 s). For this application, 2.3 s was used, which allowed a stable current plateau after the $P2X_3$ mediated peak, without compromising the overall stability of the assay.

The success rate of the assay was high and 356 out of 384 sites pass the filter criteria (93%, Figure 1B). The following filters were applied to all sites: Current amplitude ≥ 1 nA per site (sum of ten cells), whole cell capacitance ≥ 50 pF per site (sum of ten cells) and baseline seal ≥ 20 MΩ.

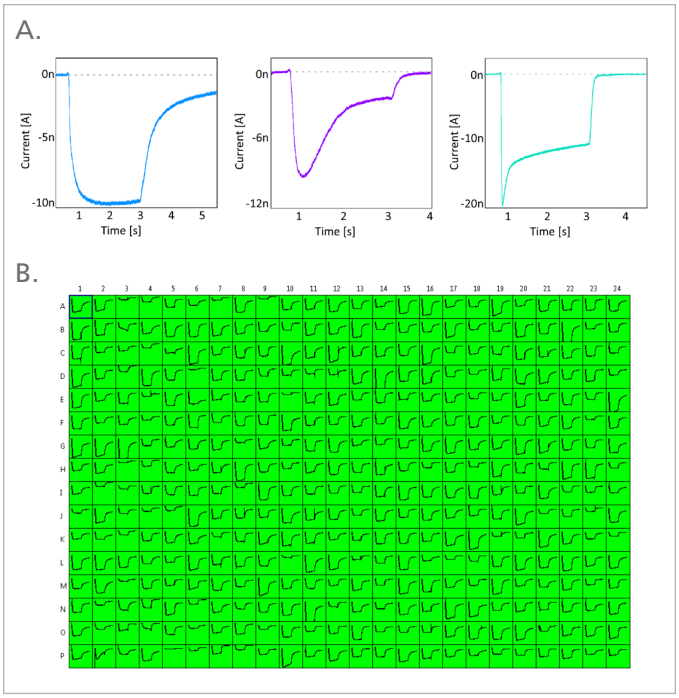

Fig. 1: A) Three responses to 10 µM ATP displaying different receptor kinetics. This demonstrates that both $P2X_2$ and $P2X_3$ homomers as well as $P2X_{2/3}$ heteromers are expressed in the cells. QChips with ten patch holes per recording site were used, summing the current from ten cells and still, a variation in response between recording sites could be seen. B) Response from exposure to 10 µM ATP for 2.3 seconds. The scale is -30 nA to 1 nA.

The agonist exposure time was 2.3 seconds, and two saline washes were performed between each addition to reduce desensitization and elude the use of hexokinase. To avoid the peak current of the rapidly desensitizing $P2X_3$ homomer, the stable current two seconds after ligand addition was used for analysis. The three agonists were used in a cumulative dose-response assay to determine the EC_{50} and EC_{80} values of ATP, αβmeATP, and CTP (see Table 1 and Figure 2). All agonists were prepared as a threefold dilution with 30 µM as the highest concentration.

Table 1: EC_{50} and EC_{80} values of the different agonists found by cumulative addition.

Agonist	EC_{50} [µM]	EC_{80} [µM]
ATP	0.53±0.3	2.16±2.0
CTP	7.20±3.1	16.68±5.6
αβ-methylene ATP	4.68±1.1	15.46±2.8

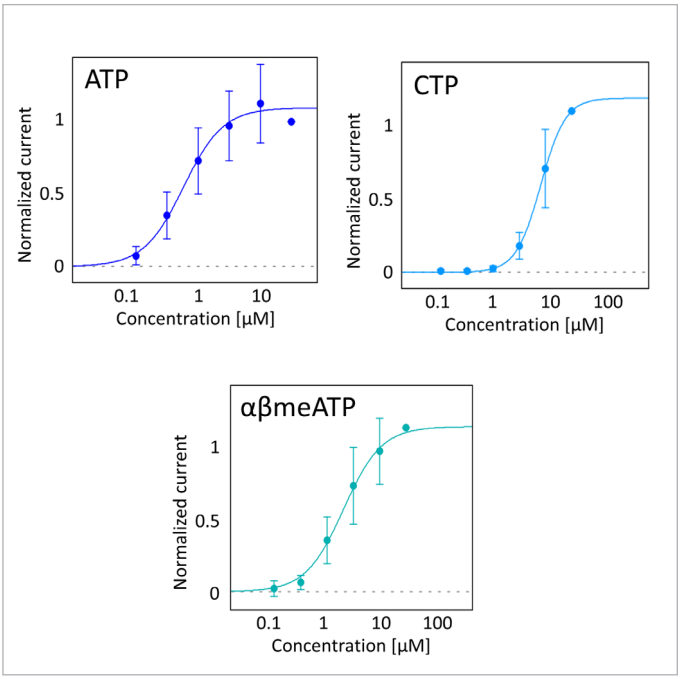

Fig. 2: Cumulative concentration-response relationship of ATP, CTP and αβmeATP binding to the $P2X_{2/3}$ receptors: Steady-state currents are normalized to the highest value in the experiment. The data is shown as average ± SD, n=48.

High stability

To test the assay stability, an agonist was added multiple times at the same concentration with two saline washes between each addition. The developed assay showed high stability and only minor rundown (see Figure 3 and Table 2). In each experiment, the first two agonist additions were disregarded in the analysis because the current level needs to stabilize.

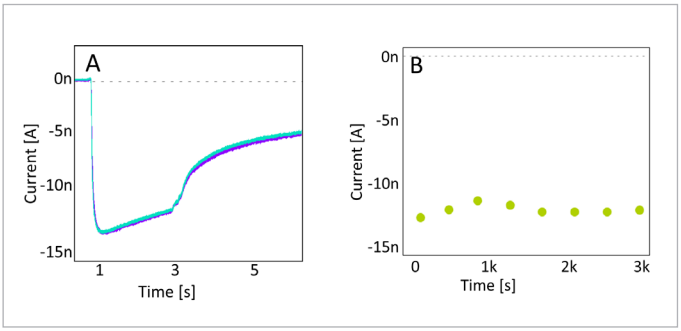

Fig. 3: $P2X_2$ and $P2X_3$ receptors have fast and extensive desensitization, which can cause severe rundown. Here we show an assay with high stability, high success rates and low run down. A) Current trace recording from 3rd and 8th liquid addition. B) A plot of current amplitude over the time course of the experiment.

Table 2: Percent average rundown from 3rd to 8th addition of 10 µM agonist. n = number of cells tested.

Agonist	Rundown [% per minute]	n
ATP	0.16 ± 0.5	20
CTP	0.25 ± 0.7	24
αβ-methylene ATP	0.39 ± 0.7	21

Pharmacology

Due to the assay's high stability, antagonists could be added cumulatively, thus increasing the number of data points per plate significantly. The three antagonists AF-353, A-317491 and suramin, were used in four increasing concentrations with 7 minutes incubation time per concentration before co-addition with agonist. Agonists were used at EC_{80} values.

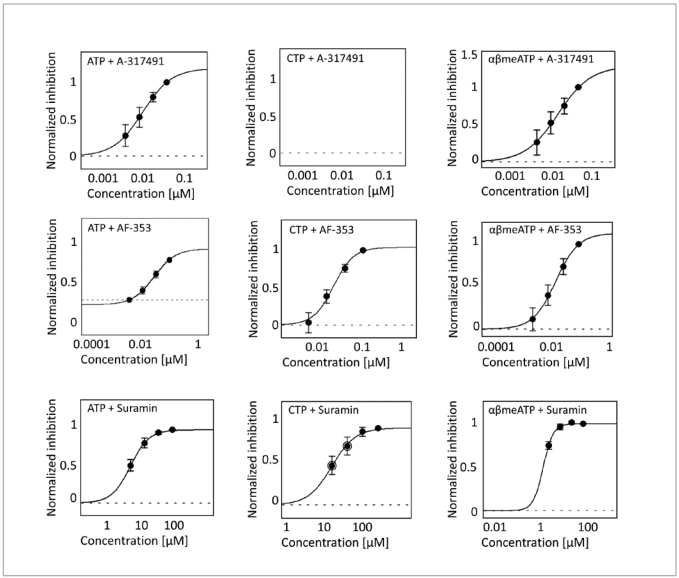

Fig. 4: Normalized inhibition of A-317491, AF-353 and suramin combined with ATP, CTP and αβmetATP. The antagonist IC_{50} values depend on the agonist they were combined with, e.g. the IC_{50} value of A-317491 varying from 55 nM with ATP to 29 nM with αβmeATP. The same pattern was seen for AF-353, which combined with ATP gave an IC_{50} value of 32 nM, 16 nM with CTP, and 18 nM with αβmeATP. The most considerable difference was found to be for suramin with IC_{50} values of 4.2 µM with ATP, 6.3 µM with CTP, and 1.2 µM with αβmeATP. All IC_{50} values were found to agree with literature values[7–9].

Table 3: The three antagonists' IC_{50} values are A-317491, AF-353, and suramin combined with the three agonists ATP, CTP, and αβmetATP. *) The concentrations of A-317491 combined with CTP did not cover a meaningful range to get a meaningful Hill fit. SD = standard deviation, n = number of experiments.

Agonist	Antagonist	IC_{50} [nM]	SD	n
A-317491	ATP	55	56	32
A-317491	CTP	*)		
A-317491	αβmetATP	29	51	28
AF-353	ATP	32	12	31
AF-353	CTP	16	3	32
AF-353	αβmetATP	18	11	31
Suramin	ATP	$4.2x10^3$	$2.3x10^3$	29
Suramin	CTP	$6.3x10^3$	$8.6 x10^3$	22
Suramin	αβmetATP	$1.2x10^3$	$0.6x10^3$	17

Note, *) The concentrations of A-317491 combined with CTP did not cover a meaning full range to get a meaningful Hill fit.

Conclusion

- $P2X_2$ and $P2X_3$ receptors have fast and extensive desensitization, which can cause severe rundown. Here we show an assay with high stability, high success rates, and low run down.
- Liquid stacking in the pipettes enables ligand exposure times down to 0.8 s. The exposure time can be precisely customized, and for this application, 2.3 s was used. The feature is essential for fast desensitizing ion channels, but it is also beneficial in cases like this, where the stable current after a specific time is of interest.
- The microfluidic channels of the QChip ensure rapid and complete solution exchange, which, together with liquid stacking results in a very stable assay without hexokinase.
- The high stability of the assay even at agonist EC_{80} values makes it suitable for cumulative dose-response experiments.
- The kinetics of the desensitization for different agonists showed that both $P2X_2$ and $P2X_3$ homomers and $P2X_{2/3}$ heteromers are present in the cells.

Methods

Cells
Experiments in this study were performed using CHO-P2X$_{2/3}$ cells, kindly provided by Axxam S.p.a. (Milan, Italy). The cells express the human P2RX2 and P2RX3 gene.

Cell Culture
CHO-P2X$_{2/3}$ cells were harvested with detachin, spun down and resuspended in serum-free medium with HEPES. For more information, contact your application scientist.

Whole-cell protocol
A two-second suction pulse from -10 mbar to -250 mbar was followed by 10 seconds at -10 mbar and after that a two second suction pulse from -10 mbar to -350 mbar was applied. For more parameters, see Figure 5.

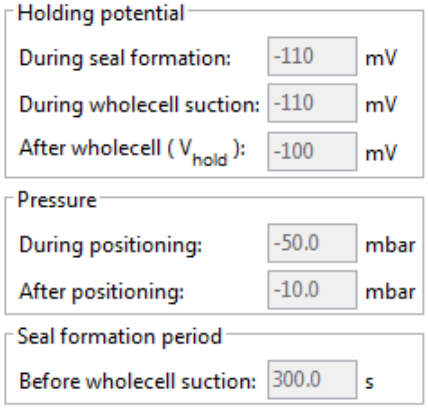

Fig. 5: Detailed parameters of the whole-cell protocol.

Experiment protocol
Cells were held at -90 mV throughout the entire experiment. Liquid stacking in the pipettes enables ligand exposure times down to 0.8 s, however, the exposure time can be precisely customized, and for this application 2.3 s was used. For more information, contact your application scientist. Each liquid application was followed by two saline washes between each addition.

Quality control filters
Current amplitude ≥ 1 nA per site (sum of ten cells)
Whole-cell capacitance ≥ 50 pF per site (sum of ten cells)
Baseline seal ≥ 20 MΩ
For cumulative dose-response experiments, Passed sweeps ≥ 8

Data Analysis
In each experiment, the first two agonist additions were disregarded in the analysis because the current level needs to stabilize.

IC_{50} values were estimated, using the "Normalized Group Hill Fit with Error" method. All results and figures are average values ± Standard Error (SD), n = number of QChip sites passed QC filters.

References:

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