

Activation and inhibition of P2X₃ channels recorded on the SyncroPatch 384PE

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Summary

P2X receptors are ligand-gated ion channels that open in response to extracellular ATP. They are permeable to small monovalent cations, some having significant divalent or anion permeability. P2X receptors are found on many cell types including smooth muscle cells, sensory neurones, epithelia, bone and leukocytes (for reviews see Refs 1 - 3). A role for P2X receptors has been suggested in transmission of thermal stimuli⁴, chemosensory signalling⁵, taste^{6,7} and pain^{8,9}. To date, 7 P2X receptor genes have been cloned and studied in heterologous expression systems (reviewed in Refs 1 - 3). Functional receptors are trimeric¹⁰, which can be homomeric or heteromeric. The P2X₂ and P2X₃ receptors can function either as homomers or as P2X_{2/3} heteromers. When expressed together, a mixture of P2X₂ and P2X₃ homomers as well as P2X_{2/3} heteromers are likely to exist, which may be distinguished through their biophysical and pharmacological properties. Both P2X₃ homomers and P2X_{2/3} heteromeric receptors have been implicated in nociception and pain signalling and may be important therapeutic targets for analgesic drugs⁸. Additionally, the P2X₃ and P2X_{2/3} receptor antagonist MK-7264 (gefapixant), has recently progressed to Phase III trials for refractory or unexplained chronic cough¹¹.

Here we present data collected on the SyncroPatch 384PE showing activation and inhibition of P2X₃ currents expressed in CHO cells with rapid and brief application of ligand ('Ligand Puff'). ATP or $\alpha\beta$ -methylene ATP ($\alpha\beta$ -MeATP) activated P2X₃ receptors with an EC₅₀ value similar to values found in the literature^{2,3,12}. P2X₃ receptors could be repetitively activated by ATP and blocked by A-317491 with an IC₅₀ value in good agreement with the literature¹³.

Results

P2X₃ expressed in CHO cells was activated by adding ATP or $\alpha\beta$ -MeATP using the Ligand Puff function in Patch-Control 384. A single concentration of ATP or $\alpha\beta$ -MeATP was applied to each well, this was repeated 3 times and finally a maximum concentration (1 mM) ATP or $\alpha\beta$ -MeATP was added to each well. The currents were normalized to the maximum current in each well and the concentration response curve calculated across the whole plate. The data was fitted using a Hill equation revealing an EC₅₀ = 2.04 μ M (61-63 wells per concentration; 372 wells in total), for ATP in excellent agreement with the literature^{2,3,12} (Figure 1).

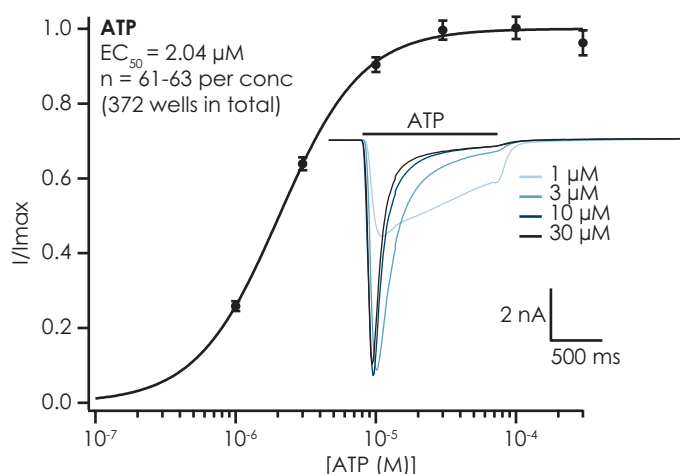


Figure 1: Concentration response curve for ATP activation of P2X₃ expressed in CHO cells. Single concentrations of ATP were added to each well followed by a saturating concentration of ATP (1 mM) to calculate the maximum peak amplitude. The data was normalized to the maximum for each well and the average concentration response curve constructed across the whole plate revealing an EC₅₀ of 2.04 μ M. Average traces are also shown in the inset.

Application Note

Currents mediated by $P2X_3$ were also activated by $\alpha\beta$ -MeATP (Figure 2) with an $EC_{50} = 1.89 \mu M$, in excellent agreement with the literature^{2,3,12}. Given the fast desensitizing kinetics of the currents, coupled with the activation by $\alpha\beta$ -MeATP, we can confirm that the currents are mediated by $P2X_3$ receptors since $P2X_2$ homomeric receptors are insensitive to $\alpha\beta$ -MeATP¹⁻³.

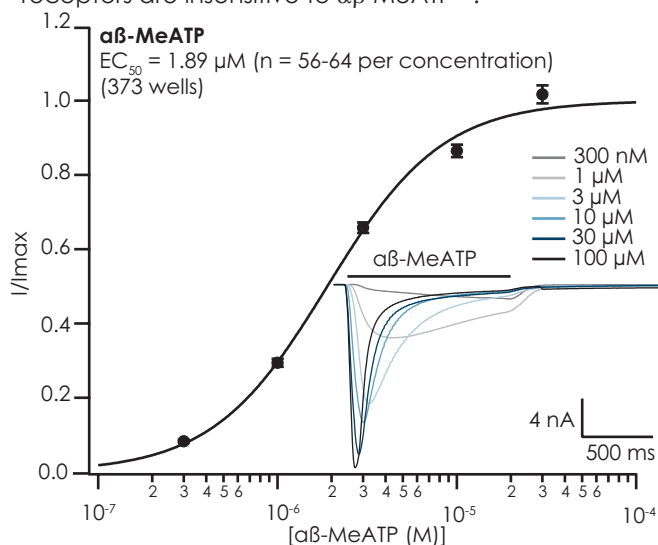


Figure 2: Activation of $P2X_3$ expressed in CHO cells on the SyncroPatch 384PE by $\alpha\beta$ -MeATP. Single concentrations of $\alpha\beta$ -MeATP were added to each well followed by a saturating concentration of $\alpha\beta$ -MeATP (100 μM) to calculate the maximum peak amplitude. The data was normalized to the maximum for each well and the average concentration response curve constructed across the whole plate revealing an EC_{50} of 1.89 μM . Average traces are also shown in the inset.

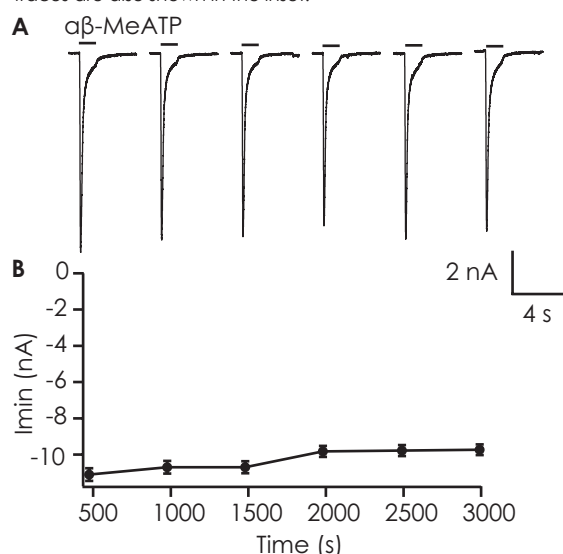


Figure 3: Stability of $P2X_3$ recorded on the SyncroPatch 384PE. **A.** Raw current traces from an example cell showing current activation by application of 10 μM $\alpha\beta$ -MeATP repeated 6 times in the same well. **B.** Online analysis plot showing peak amplitude versus time for the same well shown in **A**. The peak amplitudes were very similar with each application of $\alpha\beta$ -MeATP, differing by no more than 10% from the previous application.

For pharmacology experiments, a stable and reproducible peak amplitude is mandatory. We repetitively activated $P2X_3$ six times with 10 μM $\alpha\beta$ -MeATP (Figure 3). Peak amplitude was highly reproducible, varying with each application by no more than 10%, demonstrating the suitability of the Syncro-Patch 384PE for pharmacology experiments.

We used the $P2X_3$ selective inhibitor A-317491 to block $P2X_3$ -mediated responses. $\alpha\beta$ -MeATP was applied 3 times to check for stability followed by pre-incubation with A-317491 and then co-application of A-317491 with $\alpha\beta$ -MeATP. Single point concentration response curves, where a single concentration of A-317491 was applied to each well and the concentration response curve constructed over the whole plate, were performed. The IC_{50} for A-317491 was 92 nM in excellent agreement with the literature¹³.

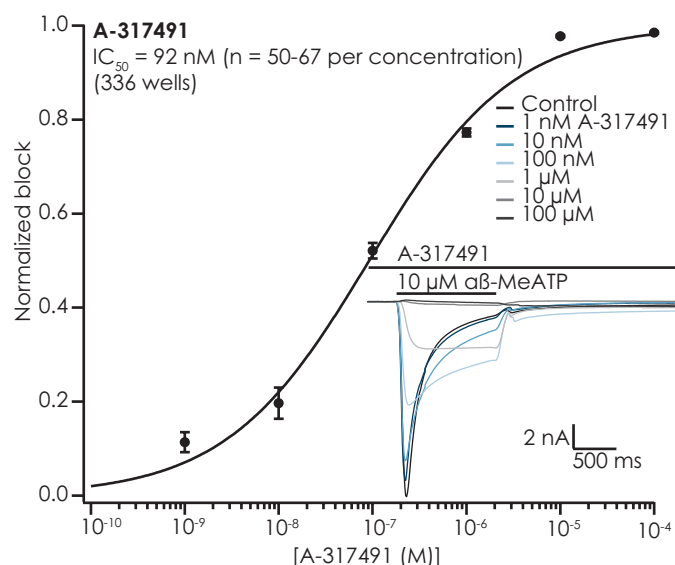
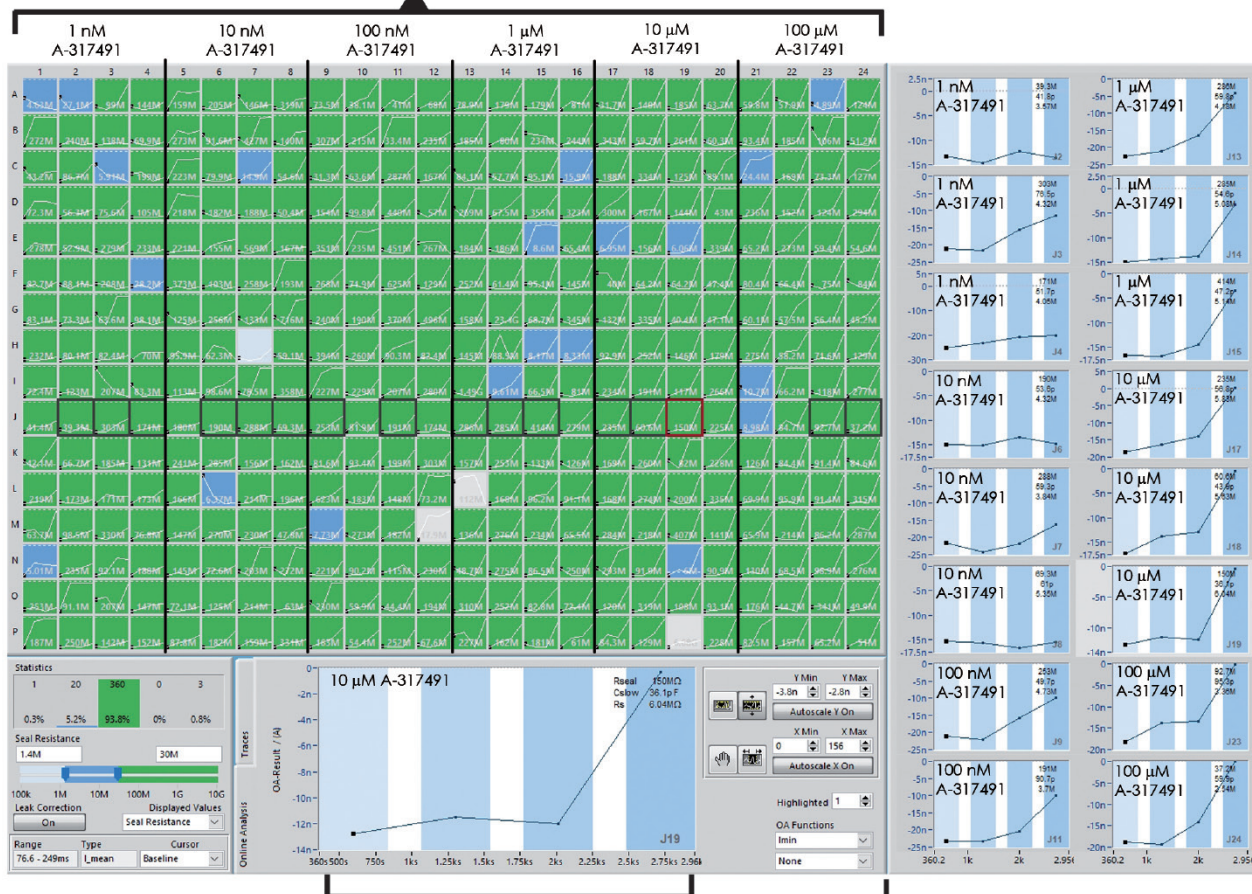


Figure 4: Inhibition of $P2X_3$ by A-317491. A-317491 was pre-incubated for 3 - 5 mins followed by co-application with 10 μM $\alpha\beta$ -MeATP. A single concentration of A-317491 was applied to each well and the average concentration response curve for all wells was constructed giving an IC_{50} of 92 nM. 336 wells were used to construct the concentration response curve giving a success rate of 88% for completed experiments.

Application Note

384 color coded depictions of data traces eases judgement of success rate



Highlighted raw traces of 1 cell showing control amplitude and effect of A-317491 on P2X₃-mediated currents

Highlighted raw traces of 16 cells showing inhibition of P2X₃ by A-317491

Figure 5: Graphical user interface of the screening and data analysis software used on the SyncroPatch 384PE. Screenshot depicts online analysis (peak amplitude) of P2X₃-expressing CHO cells as recorded on one NPC-384 patch clamp chip. At the start of the experiment, 3 control applications of $\alpha\beta$ -MeATP (10 μ M) were made 10 mins apart to check for current stability. This was followed by pre-incubation of A-317491 for 3-5 mins and then co-application of $\alpha\beta$ -MeATP and A-317491. Single concentrations of A-317491 were added to each well and the concentration response curve was calculated across the whole plate. Multi-hole chips were used where 4 holes were present per well. The data of the 384 well plate representation in the upper left part are color-coded for easy assessment of data. Depending on the seal resistance, pictures are green (Rmemb > 30 M Ω), blue (Rmemb 1.4-30 M Ω), light blue (Rmemb < 1.4 M Ω) or grey (well disabled). One highlighted experiment is displayed at the bottom, 16 selected experiments are displayed on the right. Graphs show online analysis data (peak amplitude) of P2X₃ channels following activation first by $\alpha\beta$ -MeATP followed by co-application with A-317491.

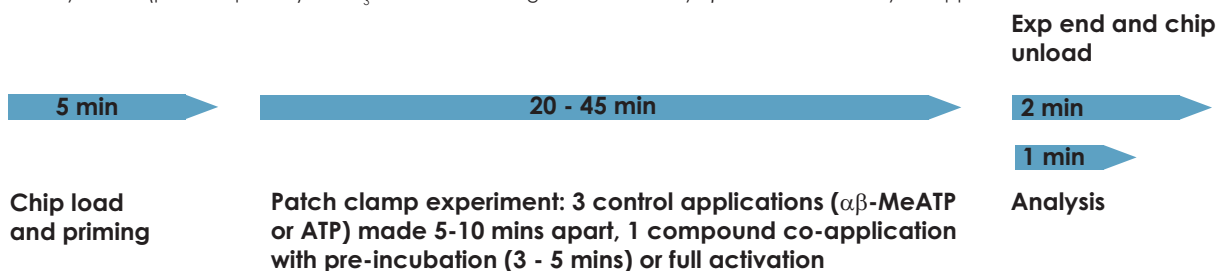


Figure 6: Timeline of an experiment on the SyncroPatch 384PE. The completion of 1 experiment on the SyncroPatch 384 patch clamp chip (384 wells) for a single point concentration response curve with control applications on P2X₃-mediated currents took approximately 27 - 45 min.

Application Note

Figure 5 shows a screenshot of the SyncroPatch 384 software during an experiment. A color-coded overview (based on seal resistance in this case) of all 384 wells gives the user a good impression of the success rate of the experiment. The user can easily toggle between raw traces and online analysis. In the example shown, online analysis values are chosen where peak amplitude in control conditions (10 μ M $\alpha\beta$ -MeATP) and when co-applied with A-317491 are shown. In these experiments, 3 control applications of $\alpha\beta$ -MeATP are performed followed by co-application of a single concentration of A-317491 in each well and the concentration response curve was calculated across the whole plate. The cells were stable for over 45 minutes, control applications were made 10 minutes apart to ensure full recovery after each application and compound was pre-incubated for up to 5 minutes ensuring that full effect of the compound was reached. An individual well can be highlighted to monitor progression of the experiment and is shown enlarged at the bottom of the screen.

In conclusion, P2X₃ expressed in CHO cells can be reliably and reproducibly recorded on the SyncroPatch 384PE. The timeline of each experiment was approximately 30 - 45 minutes (start – end) and included 3 control applications of $\alpha\beta$ -MeATP followed by co-application with an inhibitor. The inhibitor (A-317491) was pre-incubated for 3 - 5 mins and then co-applied with $\alpha\beta$ -MeATP. The P2X₃-mediated response was inhibited by A-317491 in a concentration-dependent manner with an IC₅₀ in excellent agreement with the literature¹³. The EC₅₀ values for ATP and $\alpha\beta$ -MeATP and the kinetics of desensitization were consistent with P2X₃ homomers^{1-3,12}.

The SyncroPatch 384PE is a high throughput and highly reliable automated patch clamp device for recording P2X₃ receptor-mediated currents. User-friendly software, excellent success rates, single additions or multiple additions of compound to each well and easy analysis result in reliable high quality data at an increased throughput with an economical cost per data point.

References

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Methods

Cells

CHO cells expressing P2X₃ were engineered and kindly provided by Axxam S.p.A., Milan; (<https://axxam.com/>).

Cell culture

Cells were cultured and harvested according to Nanion's standard cell culture protocol.

Electrophysiology

Whole cell patch clamp recordings were conducted according to Nanion's standard procedure for the SyncroPatch 384PE using multi-hole (4 holes per well) chips. Cells were held at -80 mV for the duration of the experiment. For ATP concentration response curves, 3U/ml hexokinase was used with higher concentrations of ATP. For pharmacology experiments, cells were pre-incubated in A-317491 for 3 - 5 min and then compound was co-applied with 10 μ M $\alpha\beta$ -MeATP. P2X₃ was activated using the 'Ligand Puff' command in PatchControl 384. Agonist volume: 3 μ l, wash volume: 7 μ l, application speed 40 μ l/s. Cells were then washed twice with external solution before re-application of agonist.