Development and validation of ligand-gated ion channel assays using the Qube 384 automated electrophysiology platform

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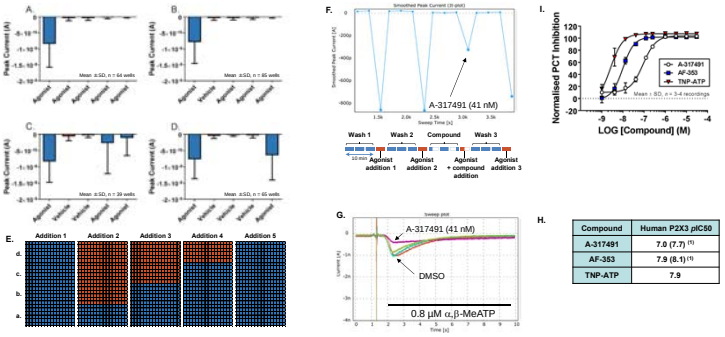
1 ABSTRACT

Ligand-gated ion channels are of particular interest to the pharmaceutical industry for the treatment of diseases from a variety of therapeutic areas including CNS disorders, respiratory disease and chronic pain. Ligand-gated ion channels have historically been investigated using fluorescence-based and low throughput patch-clamp techniques. However, with the development of the Qube 384 automated patch-clamp system, the rapid exchange of liquid and direct measurement of ion channel currents on a millisecond timescale is now possible at a greater throughput than previously possible. Here, we have used the Qube platform to develop assays against two ligand-gated families: 1) the P2X receptor and 2) the $GABA_A$ receptor families. The P2X family is comprised of 7 family members, which are cation permeable and gated by the binding of extracellular ATP. We have assessed both agonist and antagonist pharmacology of 4 members of the P2X family, $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_4$, as well as two species homologs, $rP2X_3$ and $gpP2X_3$. The $GABA_A$ α1β3γ2 receptor is a chloride permeable ion channel gated by the binding of GABA. We utilized stacked liquid addition to assess the open state kinetics of the channel and to investigate the effects of a positive allosteric modulator on channel function. As such, we have successfully characterized and developed assays for both the P2X receptor and $GABA_A$ receptor families and present EC/IC_{50} data for antagonists and positive allosteric modulators.

2 MATERIALS AND METHODS

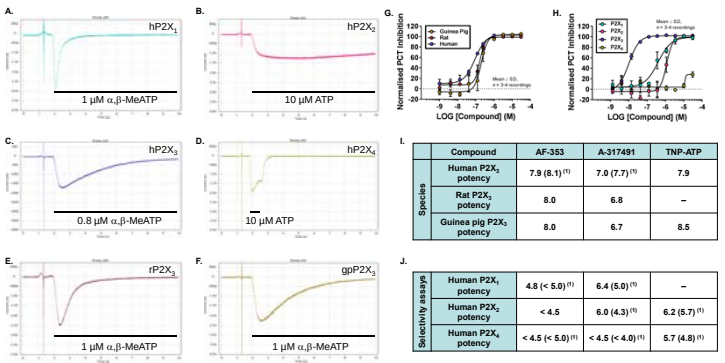
Cell Culture: HEK-$hP2X_1$, HEK-$hP2X_2$, HEK-$hP2X_3$, HEK-$hP2X_4$, HEK-$hP2X_7$, HEK-$rP2X_3$, HEK-$gpP2X_3$, and HEK-$GABA_A$ -α1β3γ2 cells were produced at Charles River Laboratories and are commercially available. All cells were grown according to their respective SOPs as developed by Charles River. Cells were kept in a serum-free medium in the cell hotel on the Qube instrument for up to 4 hours during experiment.
Solutions: For both P2X and GABA experiments the following extracellular saline solution was used (mM): 145 NaCl. 4 KCl, 10 HEPES, 10 Glucose, 1 $MgCl_2$. 2 $CaCl_2$, pH7.4. For GABA experiments the following intracellular saline solution was used (mM): 20 KCl, 120 KF, 10 HEPES, 10 EGTA, pH7.2. For P2X receptor assays the extracellular saline during wash periods was supplemented with 0.5 U/mL apyrase (Sigma) and the following intracellular saline solution was used (mM): 140 CsF, 10 NaCl, 10 HEPES, 10 EGTA pH 7.3.
Qube experiments: All experiments were carried out using the Qube platform which performs 384 parallel and independent patch-clamp recordings on a disposable, single hole or multi-hole QChip. The holding potentials used for GABA and the P2X receptor family were -70mV and -60mV, respectively.
Analysis: Data analysis was performed using Qube Analyzer software and GraphPad Prism (7.0).

3 RESULTS



Compound	Human P2X3 *p*IC50
A-317491	7.0 (7.7) [1]
AF-353	7.9 (8.1) [1]
TNP-ATP	7.9

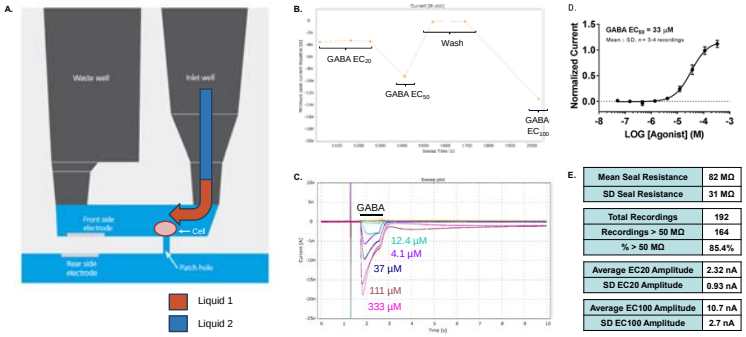
Figure 1. $P2X_3$ receptor assay development and antagonist pharmacology. *A-D.* $P2X_3$ current amplitude in the presence of vehicle/agonist. *E.* Schematic diagram of compound plates used to investigate $P2X_3$ desensitisation, indicating agonist (blue) and wash (orange) conditions. *F.* Current/time plot demonstrating inhibition by A-317491 (41 nM). *G.* Raw current trace of $P2X_3$ current in the presence of agonist ± A-317491 (41 nM). *I.* Normalized percent inhibition data with Hill fit in the presence of A-317491 (white), AF-353 (blue) and TNP-ATP (red). *H.* pIC_{50} values of each compound compared to literature values (where available).



	Compound	AF-353	A-317491	TNP-ATP
Species	Human $P2X_3$ potency	7.9 (8.1) [1]	7.0 (7.7) [1]	7.9
	Rat $P2X_3$ potency	8.0	6.8	–
	Guinea pig $P2X_3$ potency	8.0	6.7	8.5

Selectivity assays	Human $P2X_1$ potency	4.8 (< 5.0) [1]	6.4 (5.0) [1]	–
	Human $P2X_2$ potency	< 4.5	6.0 (4.3) [1]	6.2 (5.7) [1]
	Human $P2X_4$ potency	< 4.5 (< 5.0) [1]	< 4.5 (< 4.0) [1]	5.7 (4.8) [1]

Figure 2. P2X receptor family and $P2X_3$ species homolog pharmacology. Raw current traces with agonist concentration for *A.* $hP2X_1$, *B.* $hP2X_2$, *C.* $hP2X_3$, *D.* $hP2X_4$ *E.* $rP2X_3$, *F.* $gpP2X_3$. *G.* Normalized percent inhibition data with Hill fit for $hP2X_3$, $rP2X_3$ and $gpP2X_3$ in the presence of A-317491. *I.* pIC_{50} values of A-317491, AF-353 and TNP-ATP in human, rat and guinea pig P2X receptor family members compared to literature values (where available).



E.

Mean Seal Resistance	82 MΩ
SD Seal Resistance	31 MΩ
Total Recordings	192
Recordings > 50 MΩ	164
% > 50 MΩ	85.4%
Average EC20 Amplitude	2.32 nA
SD EC20 Amplitude	0.93 nA
Average EC100 Amplitude	10.7 nA
SD EC100 Amplitude	2.7 nA

Figure 3. $GABA_A$ $\alpha_1\beta_3\gamma_2$ assay development and positive allosteric modulator pharmacology. *A.* Schematic diagram of stacked liquid addition on the Qube. *B.* Current/time plot demonstrating EC_{50} and EC_{100} activation by GABA. *C.* Raw current trace of $GABA_A$ $\alpha_1\beta_3\gamma_2$ current in the presence of increasing concentrations of diazepam. *D.* Concentration response curve in the presence of increasing concentrations of GABA. *E.* Summary of recording parameters taken from 192 experiment wells.

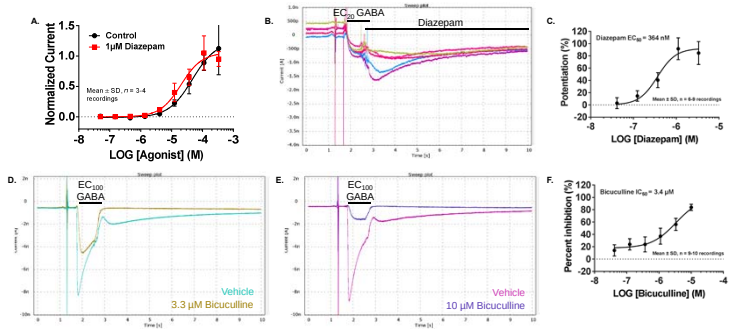


Figure 4. Assessment of positive and negative allosteric modulators using open and closed state conformations. *A.* GABA EC_{50} curve ± 1 µM diazepam. *B.* Raw current traces demonstrating modulation of GABA by diazepam in open state conformation. *C.* Potentiation of GABA EC_{20} current by increasing concentrations of GABA. *D.* Raw current trace demonstration 50% inhibition of GABA EC_{100} current by bicuculline. *E.* Raw current trace demonstrating 90% inhibition of GABA EC_{100} current by bicuculline. *F.* Inhibition of GABA EC_{100} current by increasing concentrations off bicuculline.

4 SUMMARY

Here we report pharmacological data for 4 different members of the P2X receptor family, two $P2X_3$ species homologs and the $GABA_A$ α1β3γ2 receptor using the automated Qube 384 patch-clamp system. The $P2X_3$ receptor has been shown to undergo rapid desensitization and we have demonstrated that the receptor must undergo three washes with extracellular saline to allow recovery from desensitization and return of the current to its original size. Using this three stage washing protocol, we demonstrated that $P2X_3$ currents are reproducible after multiple agonist additions and can be inhibited. pIC_{50} data for three reference antagonists was in accordance with literature values. Furthermore, a similar protocol was adopted for $P2X_1$, $P2X_2$, $P2X_4$, $rP2X_3$ and $gpP2X_3$. $rP2X_3$ and $gpP2X_3$ demonstrated comparable pIC_{50} values to that of $hP2X_3$ and literature values. In accordance with literature values, A-317491 was inactive against $P2X_4$, was less potent against $P2X_1$ and $P2X_2$ compared to $hP2X_3$.

We have developed a range of assays for investigating the effects of various types of modulators against the $GABA_A$ α1β3γ2 receptor. Using the stacked liquid addition protocol and multi-hole QChips, we demonstrated an 85% success rate (seal resistances >50 MΩ) with an average GABA EC_{20} response of 2.32 ±0.93 nA and a GABA EC_{50} concentration of 33 µM. The GABA EC_{50} curve demonstrated a leftward shift in the presence of diazepam (EC_{50} 20 µM with 1 µM diazepam). Using the open state conformation, diazepam potentiated the GABA EC_{20} current up to approximately 100% with an EC_{50} of 364 nM. The GABA current was also inhibited by bicuculline giving an IC_{50} of 3.4 µM when using an EC_{100} concentration of GABA to activate the channel.

[1] Khakh, B.S. & North. A.R. (2012) Neuron

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