

QPatch HTX



Validation of ligand-gated ion channels in multi-hole mode



We set out to test different ligand-gated ion channels that are well known and already validated on QPatch in single-hole mode to determine if the signal onset in multi-hole mode was comparable. Furthermore we investigated the effect of known compounds: agonists, antagonist and modulators to see if any changes in data quality could be detected.

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Introduction

This report summarizes the experiments that were done running fast ligand-gated assays on QPatch HTX.

QPatch HTX in multi-hole mode patches 10 cells per well instead of just one which has been shown to increase the overall success rate due to the fact that cells with less or no expression of the ion channel of interest will be patched in parallel with other cells that have better expression. The currents from all 10 holes are thus added together and therefore gives rise to increased current amplitudes from each well.

The 10 holes obviously takes up more space in the flow channel in the QPlate chip and we were curious to know if these changes had any effect on the measured signal from the ten cells.

We set out to test different ligand-gated ion channels that are well known and already validated on QPatch HT to determine if the signal onset was comparable to QPatch HTX in multi-hole mode. Furthermore we investigated the effect of known compounds: agonists, antagonist and modulators to see if any changes in data quality could be detected.

Materials & Methods

Setup

The experiments were all conducted in the course of about 2 weeks in April 2009 at Sophion Bioscience A/S in Denmark on a prototype QPatch HTX equipped with modified beta test type HTX polyamps 4.0.

Cells and solutions

The cell lines used were:

- RBL 2H3 (endogenous Kir)
- TE671 (endogenous nicotinic α1)
- HEK 293 GABAA
- RLE P2X3
- HEK GluR5
- GH4 C1 nicotinic α7

Below are the solutions that were used for all of these cells except for RBL 2H3:

Intra cellular Ringer's (in mM): 120 KF, 20 KCl, 10 HEPES, 0.1 EGTA, Osmolarity 275 mosm, pH 7.2.

Extra cellular Ringer's (in mM): 145 NaCl, 4 KCl, 10 HEPES, 1 MgCl₂, 2 CaCl₂, Osmolarity 295 mosm, pH 7.2.

For RBL 2H3 we used the same extracellular Ringer's but the intracellular Ringer's was 120 KCl, 10 HEPES, 10 EGTA, 1.75 MgCl₂, 5.4 CaCl₂.

All experiments were done using glass vial compound plates.

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Results

RBL2H3

This cell line has been widely used to answer different flow and pipetting related questions. The protocol used is shown below in figure 1a and a typical response is shown in figure 1b.

RBL 2H3 expresses an inward rectifier in large amounts and changing driving force for K will induce a "ligand"-gated response that can be analysed.

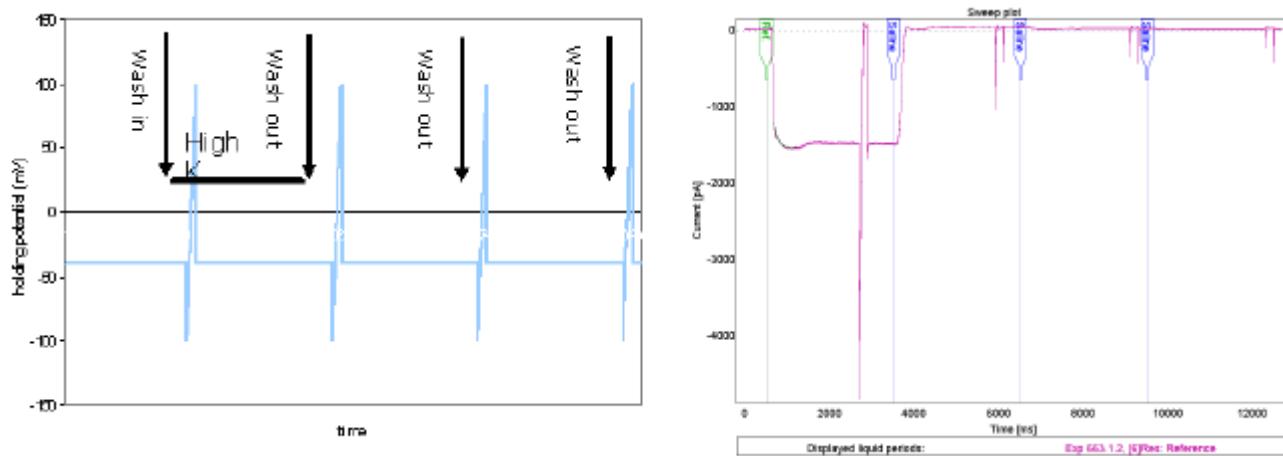


Figure 1. Left: Voltage protocol including pipetting time stamps. High K is added for 3 sec. and then washed off again. The protocol includes 4 ramps where K concentration in the flow channel can be estimated. Right: Typical response from a single cell experiment. The bottom of the ramp is highlighted with black dots. It can be seen that three washes is needed for complete washout.

The exchange rate was evaluated in two ways in this set of experiments. By adding a 10-90 % rise time cursor and by fitting the "response" to a single exponential function (see Figure 2 below).

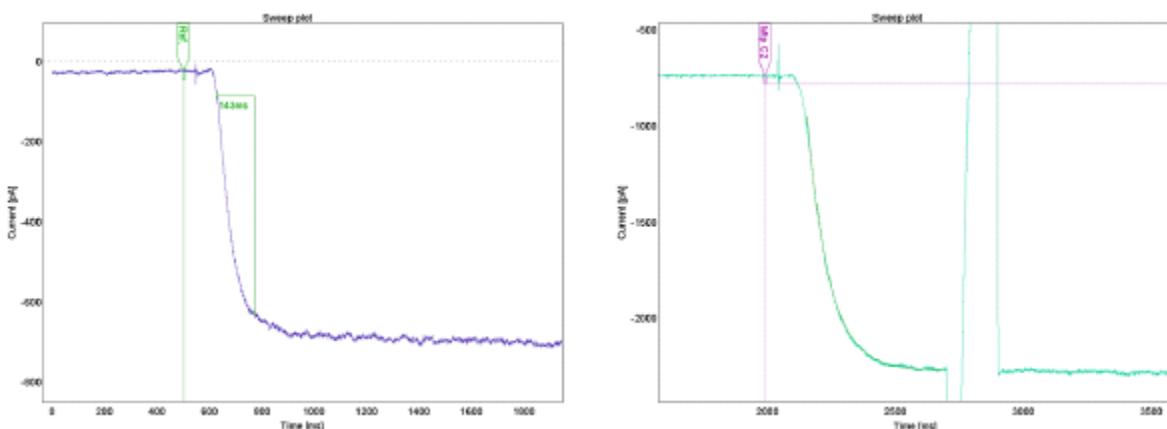


Figure 2. Left: 10 – 90 % rise time cursor on a single-hole experiment. Right:RBL response recorded in multi-hole mode and fitted to $A + B\exp(-Ct)$.

The data from two inserts collected from single-hole and multi-hole experiments are summarized in Table 1

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below.

It was found that the current amplitude was increased from 0.7 to 5.5 nA i.e. app. 8 fold when comparing single-hole and multi-hole. This indicates that we have close to, but not full whole cell efficiency when running multi-hole.

When comparing the rise time parameters between the two systems it was found that there was no difference in this regard. The rise time was 170 ms for single hole and 183 ms for multihole ("HTX") and tau was estimated to be 89 and 69 respectively.

	Single-hole	HTX
Current response (nA)	0.7 +/- 0.3	5.05 +/- 2.0
Rise time (10 - 90%) (ms)	170 +/- 49	183 +/- 27
"Activation" 1/C (ms)	89 +/- 18	65 +/- 14

Table 1. Current response, rise time and activation time constant measured on RBL 2H3 with single-hole and multi-hole ("HTX") measurement plates.

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TE671 nicotinic acetylcholine receptor $\alpha 1$

We tested rise times on the acetylcholine sensitive nicotinic $\alpha 1$ channel in TE671 cells. This cell line expresses this channel endogenously and is a more relevant assay since $\alpha 1$ is a "real" ligand target especially used for counter screening.

This could potentially be an assay where a slower rise time could also be detected. This time we looked at the rise time as a function of increasing concentrations of acetylcholine. We ran the same assay on QPatch HTX in multi-hole and single-hole mode and compared the 10 – 90 % rise time in the same manner as above in the RBL 2H3. The results are summarized in Figure 3 below.

It can be seen that the rise time decreases as a function on acetylcholine as expected - from app. 150 ms to 25 ms on the highest concentrations. It was found that there was no difference between single-hole and multi-hole when comparing rise times on the two modes.

The success rate was found to be good in both systems: 90 and 93 % sites with $\alpha 1$ currents were found for single-hole and multi-hole mode respectively.

This success rate is counted from a total insert i.e 16 sites or more also counting sites that does not prime, gets a cell positioned or fails in another way. Hence 90% means that 90% of all sites obtained whole cell configuration with measurable $\alpha 1$ current in single-hole mode.

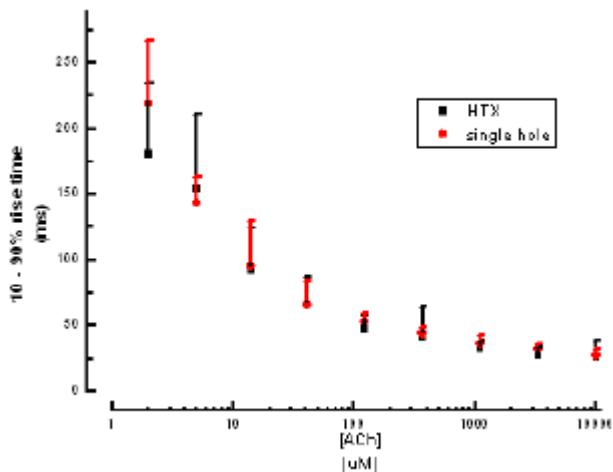


Figure 3. Average 10 – 90% rise times +/- SD from at least 16 sites measured as a function of acetyl choline concentration in HTX/multi-hole and single hole mode.

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RLE rP2X3

This ATP gated channel was the third cell line where the rise time was evaluated. This rat cell line has been used to validate ligand-gated assays in 2005 on QPatch where it was found to have a success rate of around 25 %, not because of patchability issues, but mainly due to low expression rates in the cell culture.

It was therefore interesting not only because it is one of the fastest channels of the P2X family but also because we wanted to test if we could improve the overall success rate with QPatch HTX in multi-hole mode. Below is a figure (Figure 4) showing a raw data recording of the rise time as a response to 10 mM ATP and a summarizing figure of the rise time as a function of ATP concentration.

Again, it can be seen that the rise time decreases as a function agonist - from app. 250 ms to 15 ms on the highest concentrations. It was found that there was no difference between single-hole and multi-hole mode when comparing rise times.

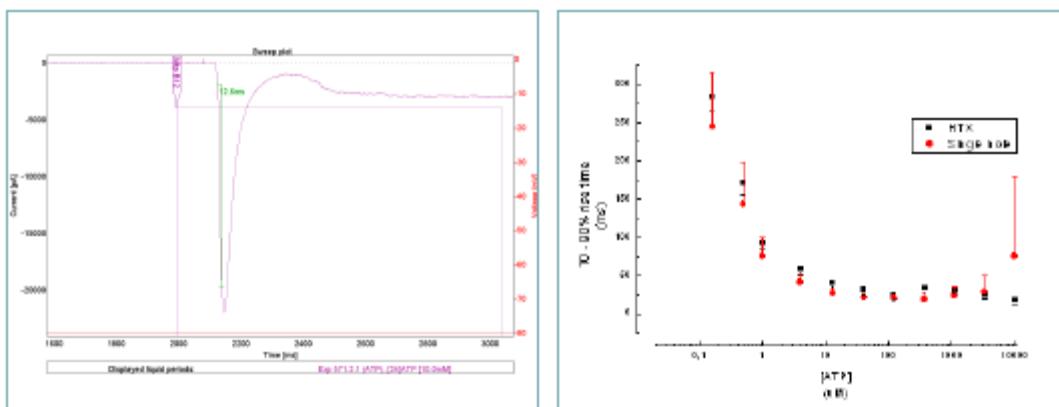


Figure 4. Left: Leak subtracted 10 mM ATP induced P2X3 response with 10 – 90 % rise time cursor. Rise time is 17 ms. Right: Average rise time +/- SD vs. ATP concentration.

Surprisingly, we found that the success rate was 81% in single-hole vs. 100 % in multi-hole mode. As stated above we had expected the success rate to be close to 25% sites with current. This increase indicates that we have become better at culturing these cells or that this assay has improved over time.

In any case we could again see that the success rate went up to 100% when running on in multi-hole mode.

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GH4 C1 nicotinic acetylcholine receptor $\alpha 7$

The nicotinic acetylcholine $\alpha 7$ channel has already been shown to be measurable on QPatch HT (Friis, Mathes et al. 2009). This target is an interesting test case for QPatch HTX in multi-hole mode for several reasons:

First of all because this channel also has a very fast onset and desensitization, but mainly because these channels are very hard to express in high enough quantities to get a usable signal (more than 50 pA) in single hole mode.

The channel was stimulated with 1 and 10 mM acetylcholine and the rise time was compared to single hole data with the same protocol. Figure 5 a & b below shows raw data sweeps obtained with GH4 C1 stimulated with acetylcholine.

The dataset was acquired twice – first time with cells that had been frozen down for about 1½ years. These particular vials contained cells from the previous optimization project on this cell line. A second data set was done with cells that were not optimized.

The results from the two data sets are summarized in Table 2 below.

	Single-hole	HTX	Cell type
Peak current @ 1 mM ACh (pA)	-114 +/- 32	-460 +/- 328	Optimized cells
% sites with $\alpha 7$ current	25%	81%	Optimized cells
Peak current @ 1mM ACh (pA)	-33 +/- 23	-136 +/- 69	Non-optimized cells
% sites with $\alpha 7$ current	6%	28%	Non-optimized cells

Table 2. Current amplitude and success rate evaluated with two different batches of GH4 C1 cells in single-hole and multi-hole ("HTX") mode.

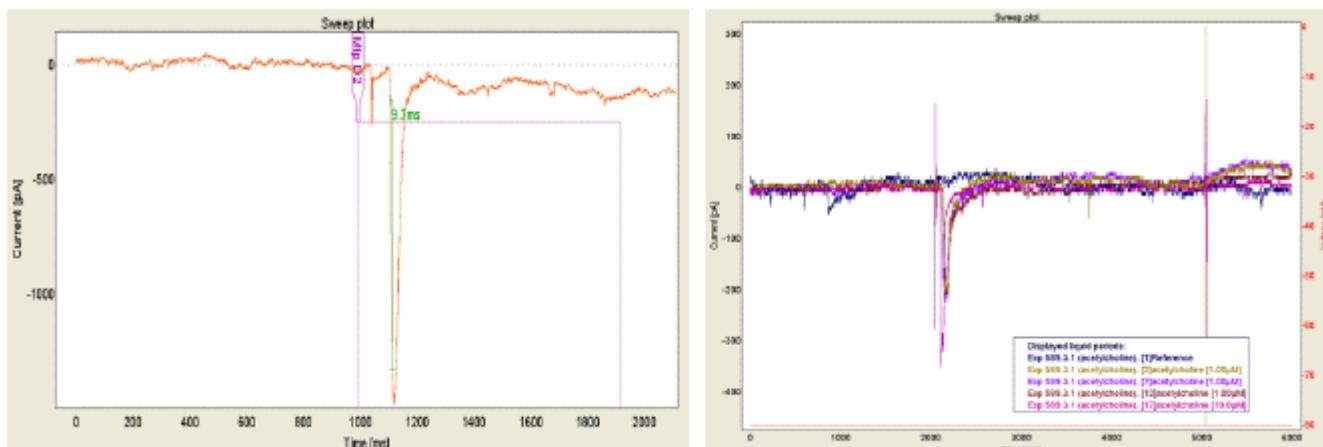


Figure 5. Left: Leak subtracted non optimized cells stimulated with 3x1 mM and 1x 10 mM Ach. Right: Leak subtracted optimized cells stimulated with 10 mM ACh.

It can be seen that running the experiments with QPatch HTX technology increases the success rate with both cell types. It was found that the optimized cell line performed better than the non-optimized cell line as expected. The success rate with the optimized cell is increased from 25 % to 81%, which for this particular cell line is considered very good. When running non-optimized cells the success rate is also increased but this time from 6% to 28%. The rise time was found to be the same when comparing the two systems (data not shown).

Pharmacological Data

To further evaluate these ligand-gated assays on QPatch HTX we performed a number of tests with known agonists, antagonists and modulators of these channels.

The protocols that were used for these experiments are all standard protocols for ligand-gated assays. The cycle interval is 150-200 seconds between each round and this is also the length of preincubation used when compounds has been tested together with an agonist. In some assays enzymatic wash is used between each cycle – yielding 5 test pt. per well. Assays without enzymatic wash yielded 6 pt. per well.

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rP2X3

ATP, $\alpha\beta$ - met-ATP and TNP-ATP was tested on rP2X3. The results can be seen in Figure 6 below. The number of repeats of each experiment type is in no case less than 4. The success rates in these experiments were found to be equivalent to the initial rise time experiments. Figure 6 below shows raw data sweeps obtained with GH4 C1 stimulated with acetylcholine.

XC50 of known compounds on rP2X3 (uM)			
	Single-hole	HTX	Lit. values
ATP	0.7	1.5	1.8 (Wildman, Brown et al. 1999; Wildman, King et al. 1999)
$\alpha\beta$ - met-ATP	1.5	0.3	2.7 (Pratt, Brink et al. 2005)
TNP-ATP @ 1 μ M $\alpha\beta$ - met-ATP	0.021	0.029	0.009 (Virginio, Robertson et al. 1998)

Table 3. EC₅₀ values for the agonists ATP and ab-met-ATP and IC₅₀ for the antagonist TNP-ATP obtained on P2X3 compared between multi-hole ("HTX"), single-hole, and literature values.

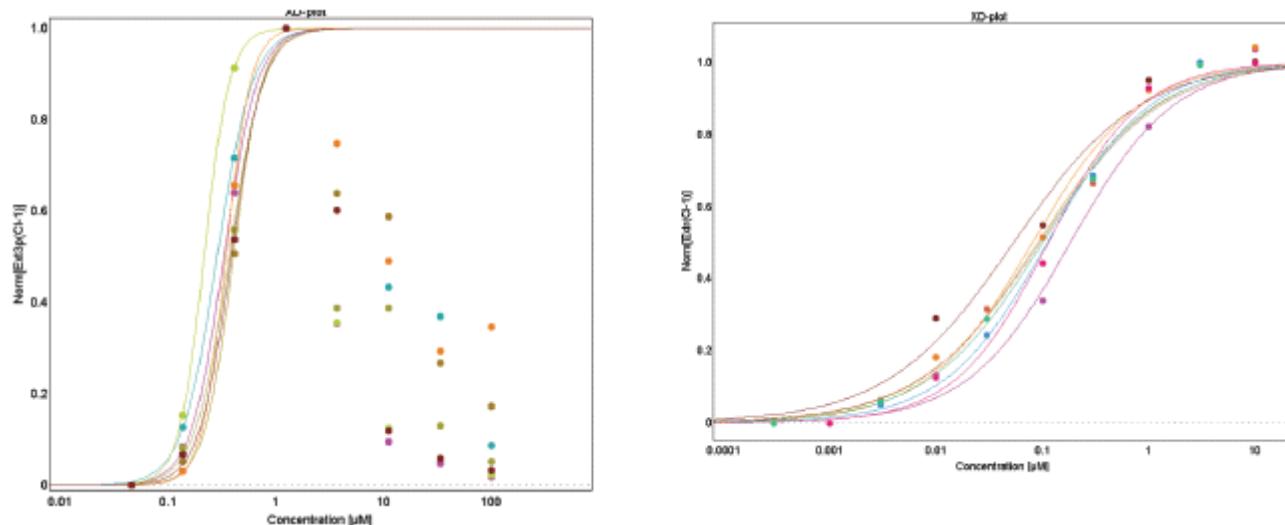


Figure 6. Left: Normalized $\alpha\beta$ - met-ATP rP2X3 peak responses vs. concentration from 4 different cells. The responses from concentrations eliciting increasing responses is fitted to the Hill equation to estimate EC₅₀ Right: Normalized TNP-ATP induced block. Cells stimulated with 1 μ M $\alpha\beta$ - met-ATP. Data combined from 4 different cells.

The data that was collected with QPatch HTX was found to be comparable to reference data from both QPatch HT and also from literature references.

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GABA_A

GABA $\alpha 1\beta 2\gamma 2$ expressed in HEK293 cells was also one of the cell lines tested in this validation study.

The reason for this is that this particular cell line has previously been shown to have an effective success rate of around 40% mainly due to low expression of the ion channel.

GABA is also a widely used ion channel in transient expression assays due to the many possible combinations of subunits. The success rate for transfections is typically also around 40% and hence this would be an assay that would be well suited for running on QPatch HTX in multi-hole mode.

The results are summarized in Table 4 and Figure 7 below.

EC ₅₀ of known compounds on GABA (uM)			
	Single-hole	HTX	Lit. values
GABA	5.6	12	9-18 (Curtis, Duggan et al. 1970)
Bicuculline	1.2	1.5	1-3 (Curtis, Duggan et al. 1970)
Diazepam @ 1 uM GABA	ND	0.14	0.15 (Kapur and Macdonald 1996)
% sites with current	37%	93%	

Table 4. EC₅₀ values for GABA, the positive modulator diazepam and IC₅₀ for bicuculline compared between multi-hole ("HTX"), single-hole, and literature values.

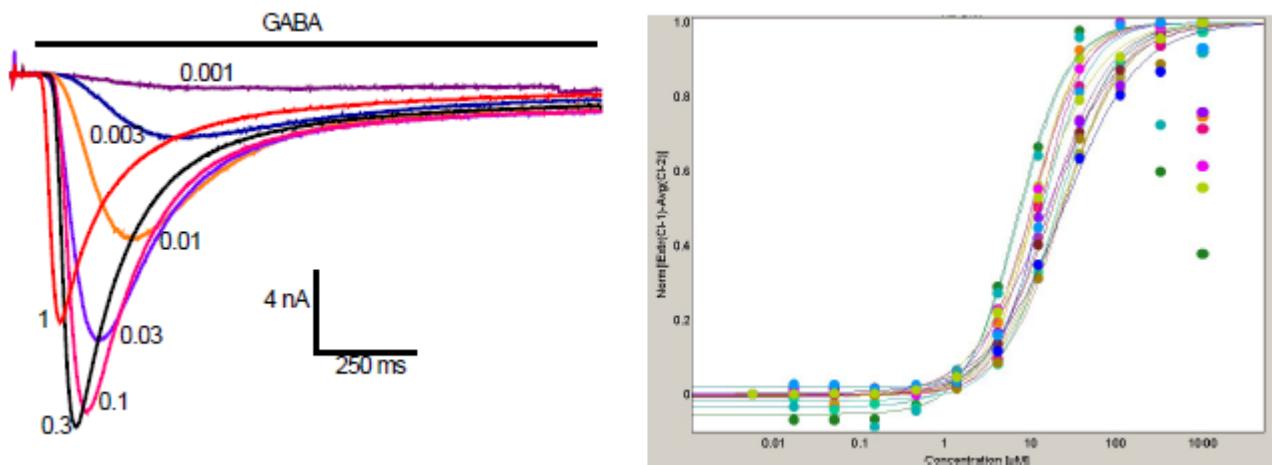


Figure 7. Left: Leak subtracted GABA $\alpha 1\beta 2\gamma 2$ currents recorded as response to increasing concentrations of GABA (mM). Right: Normalized GABA responses from 16 individual sites fitted to the Hill equation. Each cell was exposed to 12 different concentrations of GABA.

It can be seen that the success rate indeed was increased from 37% (as expected) in single-hole mode to 93% sites with GABA current seen in multi-hole mode. The pharmacological data recorded on GABA was found to be very close to what has been seen in single-hole mode and what was found in the literature.

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Nicotinic Acetylcholine Receptor $\alpha 1$

This channel is affected by local anesthetics and a number of these were tested. The cells were again preincubated with the compound before a dose of acetyl choline (1 mM) was added together with the test concentration of compound. The results are shown in Table 5 and Figure 8 below.

EC ₅₀ of known compounds on nicotinic $\alpha 1$ (uM)			
	Single-hole	HTX	Lit. values
Acetyl choline	5.6	12	8.5 (Shao, Mellor et al. 1998)
Tetracaine	ND	1.6	13 (Gentry and Lukas 2001)
Lidocaine	ND	<100	52 (Gentry and Lukas 2001)
Gallamine	5.3	2.2	0.9 (Paul, Kindler et al. 2002; Purohit, Tate et al. 2007)
Rocuronium	ND	0.13	0.33 (Paul, Kindler et al. 2002; Purohit, Tate et al. 2007)
% sites with current	90%	93%	NA

Table 5. EC₅₀ values for agonist acetylcholine and IC₅₀ for four known antagonists and success rates obtained with TE 671 on single hole, multi-hole ("HTX") and literature values.

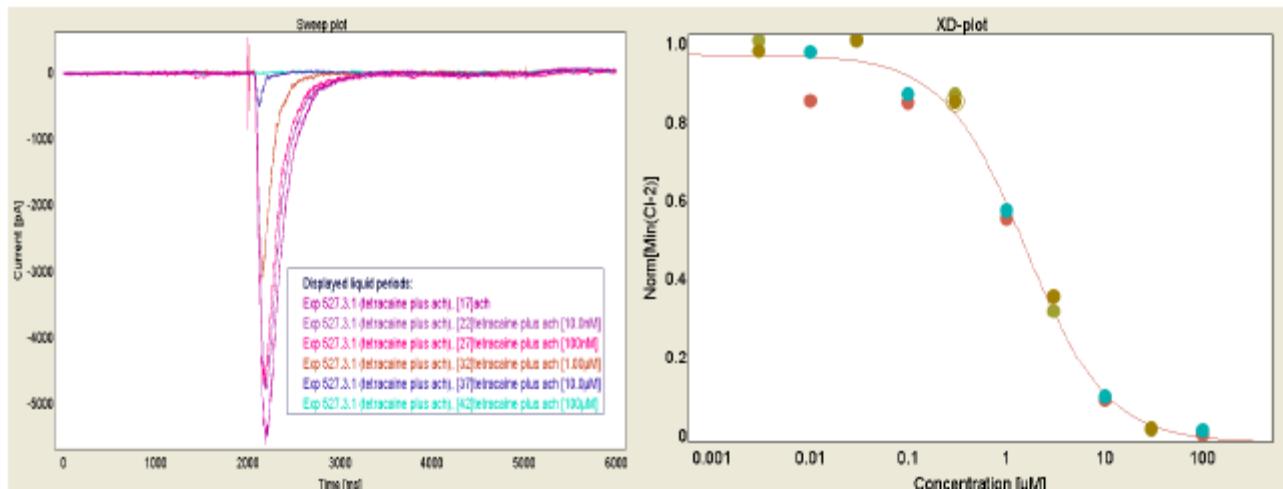


Figure 8. Left: $\alpha 1$ current stimulated with 1 μ M ACh in the presence of increasing concentrations of tetracaine (0.01 – 100 μ M). Right: Resulting concentration response plot from 4 sites, IC₅₀ = 1.6 μ M for tetracaine.

Again we found excellent agreement between data obtained on QPatch HTX and QPatch HT single hole for all of these compounds tested. The success rate was not increased tremendously but this is due to the fact that this cell line performs very well in both single-hole and multi-hole mode – around 90% sites with current in each.

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GluR5

This cell line was also tested in multi-hole mode and compared to single-hole data obtained previously. The channel is expressed in HEK 293 cells. The results of the tests with its natural agonist glutamate, the analogue kainate and an antagonist are shown below in Table 6 and Figure 9.

XC50 of known compounds on GluR5 (uM)			
	Single-hole	HTX	Lit. values
Glutamate	236	344	630 (Lerma, Paternain et al. 2001)
Kainate	119	299	33-177 (Lerma, Paternain et al. 2001)
CNQX	1.9	0.8	
% sites with Kainate current	65	91	

Table 6. EC₅₀ values for agonists and IC₅₀ values for antagonists tested on GluR5 and compared between HTX, single hole and literature values.

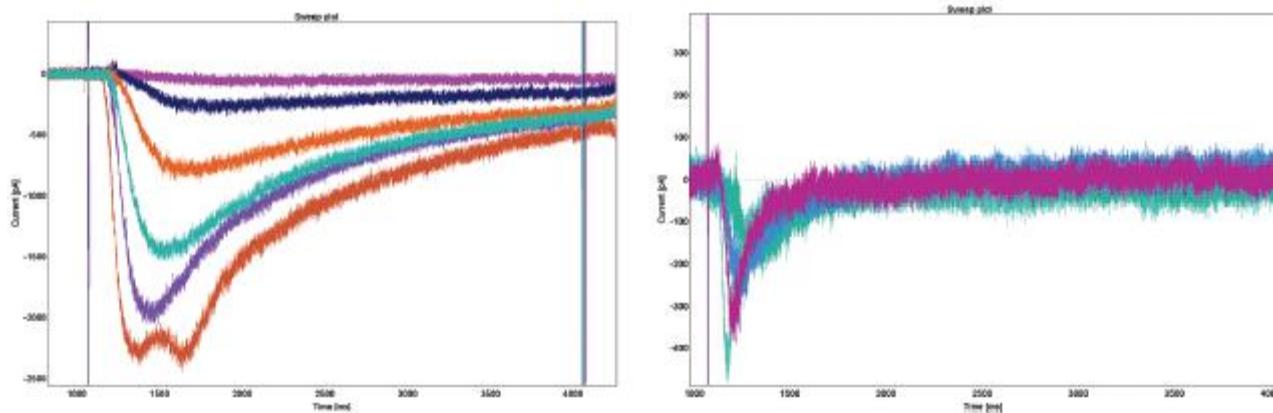


Figure 9. Left: Leak subtracted GluR5 currents recorded as response to increasing concentrations of kainate. Right: Leak subtracted GluR5 currents recorded as response to increasing concentrations of glutamate.

With this cell line we again saw a marked increase in success rate when evaluating sites with kainite induced currents. The current response was found to be markedly increased when using kainite as the agonist compared to using glutamate which has also been seen on regular QPatch HT. The response was found to be 10-fold increased when running HTX.

With this assay QPatch HTX could facilitate the use of glutamate which is the natural ligand for this channel since the currents increased to a level where it is usable. However it is still recommended to use kainate since the signal is larger and more stable with this ligand.

The results XC50's found on this channel with QPatch HTX are comparable to the data found on QPatch HT and when compared to literature values.

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References

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Conclusion

This study investigates the use of fast ligand-gated applications on QPatch HTX in multi-hole mode in order to evaluate these types of demanding channels with this technology.

We evaluated the rise times of rP2X3, nAchR $\alpha 1$ and $\alpha 7$ and found that these characteristics were the same when compared to single-hole recordings.

In addition we also tested a number of known reference compounds to validate the pharmacology on these channels. The results found on GABA A , P2X3, nAchR $\alpha 1$ and GluR5 were all found to be in very good agreement with data obtained in single-hole recordings.

It was seen that running these assays in multi-hole mode increased the success rates in all cases. In most assays the success rate was at least 90%. For non-optimized nAchR $\alpha 7$ it was found to be 28%, but still increased from 6%.

We therefore conclude that QPatch HTX in multi-hole mode is indeed very well suited for running fast ligand-gated ion channels – it increases the success rate without sacrificing any of the data quality found with single-hole recordings.