

## Application Report

# GH4-C1 nAChR $\alpha 7$ - Nicotinic acetylcholine receptor $\alpha 7$ using QPatch

QPatch was used to perform experiments on GH4-C1 cells expressing nAChR  $\alpha 7$

### Summary

This study presents results produced on QPatch with GH4-C1 cells expressing the homomeric nicotinic ligand-gated  $\alpha 7$  ion channel (nAChR  $\alpha 7$ ). The study was performed in collaboration with Wyeth Research

QPatch was used to perform experiments on GH4-C1 cells expressing nAChR  $\alpha 7$  and here are some key findings:

- GH4-C1 cells sealed well
- QPatch can detect and measure  $\alpha 7$  current
- QPatch nicely reproduce Dynaflo data e.g. partial agonism
- QPatch is suited for Positive Allosteric Modulators (PAM), blocker and agonist characterization

### Introduction

This study presents results produced on QPatch with GH4-C1 cells expressing the homomeric nicotinic ligand-gated  $\alpha 7$  ion channel (nAChR  $\alpha 7$ ).

nAChR  $\alpha 7$  is linked to many normal and pathological conditions. It is important in memory formation, arousal, and attention, nicotine addiction and nicotine-induced reversal of age-related memory deficits. nAChR  $\alpha 7$  activation leads to neuroprotection against amyloid- $\alpha$ -induced neurotoxicity, which suggests that the receptor may play an important role in Alzheimer's disease. Also, their role in hippocampal auditory gating has linked the receptor to schizophrenia. These findings make this particular channel an interesting target for pharmaceutical companies.

The fast kinetics of the  $\alpha 7$  channel makes it a difficult ion channel to work with, even using conventional patch clamp rigs.

At Sophion we have developed an assay that captures nAChR  $\alpha 7$  current in a reproducible way enabling automated technology to be used in the search for blockers and positive allosteric modulators of this channel.

### Results

#### Sealing rates

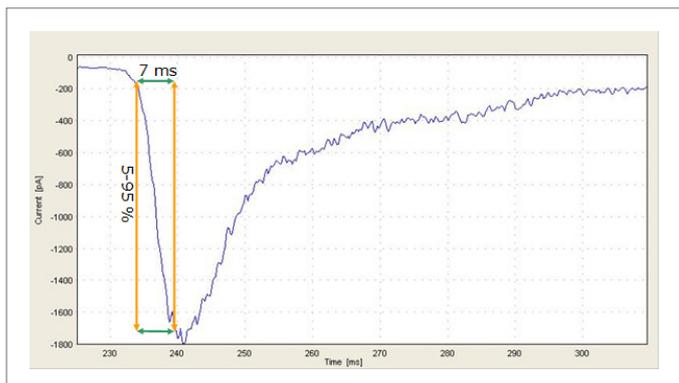
The sealing rate with GH4-C1 cells is generally good, however, their small size poses a challenge for gaining whole-cell access. It is not uncommon to see cell capacitances between 2 and 4 pF. In some of these experiments, clear single channel activity has been seen (see below).

The whole cell rate was about 80% with this cell line, however the final success rate with useful experiments was significantly low due to the lack of channel expression in some cells.

#### Signal detection

The first set of experiments comprised simple detection of measurable  $\alpha 7$  current. This particular ion channel is known to activate after stimulation with agonist and then desensitize in less than 10 ms. We have previously shown that we have an exchange rate of potassium of around 60 ms in the QPlate so we were curious to see if we could detect any signal at all. On the other hand, this channel is regularly investigated in *Xenopus* oocyte set-ups where the flow in the chamber and the size of the oocyte is significantly influencing the measured signal.

We tried different settings and concentrations of acetylcholine (ACh) and found that we could clearly see  $\alpha 7$  current. An example of a trace is shown in Figure 1.



**Fig. 1.**  $\alpha 7$  response to 10 mM Ach. The rise time was found to be between 5-10 ms for 10 mM Ach.

It was surprising to us that we were able to see rise times around 10 ms. This is somewhat slower than what you would expect from a manual rig with a pizo stepper where the rise time can be approx. 1 ms at high concentrations.

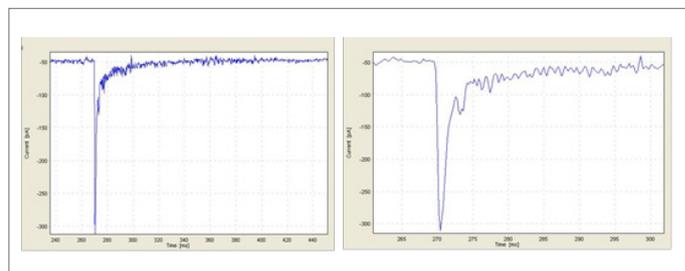
The discrepancy between our previous potassium measurements where we have seen much slower exchange rates ( $\tau = 65$  ms) and the here recorded rise time is not known. We speculate that the exchange rate between two solutions will be dominated by the “sharpness” of the liquid interfaces. The sharpness is influenced only by simple diffusion between the two liquids. The diffusion is proportional to the diffusion constant of the agonist. Since potassium is a smaller molecule than acetylcholine the diffusion constant is smaller, causing a sharper liquid interface with acetylcholine. Another parameter determining the diffusion constant of a compound is viscosity – diffusion will be less the higher the viscosity of the solution. We have made some preliminary experiments where we have raised the viscosity of the liquids with 10% ficoll. This resulted in a dramatic increase in the rise time (Figure 2). Although this approach still needs some more testing it seems very promising – we have never seen such rise times without ficoll before.

We have preliminary experiments with other rapid ligand-gated channels where ficoll also increases the peak signal.

### Stability

Alpha7 has a very tight window with regard to activation/desensitization. The amount of acetylcholine that needs to be present in order to desensitize the channel is extremely low compared to what needs to be added to activate it.

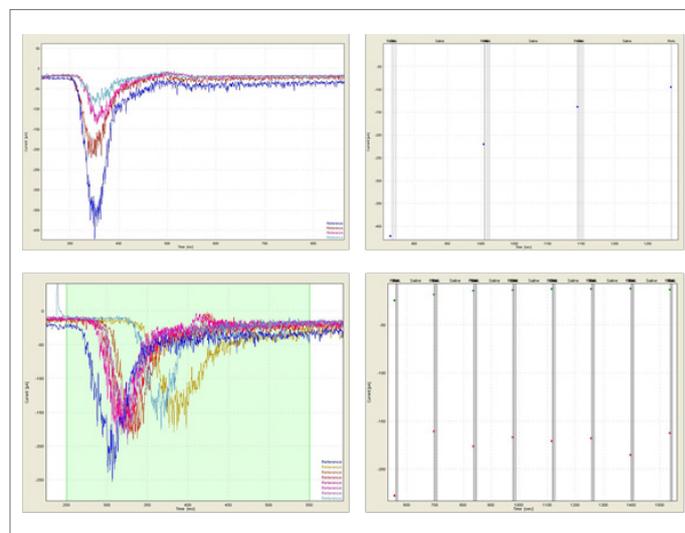
Therefore there is a challenge in getting the same signal when doing repeated additions of the same concentration of agonist. In order to use this channel in the QPatch for drug screening and drug characterization, a stable signal is obviously a key requirement.



**Fig. 2.**  $\alpha 7$  response to 300  $\mu$ M nicotine in the presence of 10% ficoll. Rise times are reduced to app. 1 ms.

This was tested by looking at the  $\alpha 7$  signal with a relatively high acetylcholine concentration (1 mM). The experiment was set up to look at the response from repeated additions of the same concentration of agonist. Normally we have no problems doing this, but we found that with  $\alpha 7$  there was a decrease in the current response during the experiment.

We then added acetylcholinesterase (5 U/mL) to the washing solution to enzymatically “clean out” the agonist. It was found that the response could be repeated several times with the same concentration of acetylcholine.



**Fig. 3** Top panel: 4 consecutive additions of 1 mM acetylcholine without acetylcholinesterase in the washing solution. Overlaid raw data on the left and I/T plot on the right. Bottom panel: 8 additions of 1 mM acetylcholine done with esterase in the washing solution.

The reason for this observation is probably due to the relatively slow off-rate of acetylcholine and the properties of the channel that prevents using the more conventional “mechanical” washing procedure that normally is employed on the QPatch. It can be speculated that the residual acetylcholine that is still bound to the receptor eventually equilibrates with the surrounding solution and thus gradually desensitizes the remaining channels to some extent. The enzyme will break down acetylcholine into acetate and choline, and since choline has a 10-fold lower affinity for the receptor, the desensitizing effect will be insignificant.

Figure 3 shows a comparison between enzyme and non-enzyme containing experiments.

### Agonist dose-response

#### Full agonism

These channels can be stimulated with a variety of agonists - we have made assays to test both full and partial agonists on the QPatch.

Acetylcholine is considered to be a full agonist for this receptor. The dose-response relationship for this agonist was tested in a set of simple 8 point dose response experiments. The raw traces from one of these experiments are shown below in Figure 4.

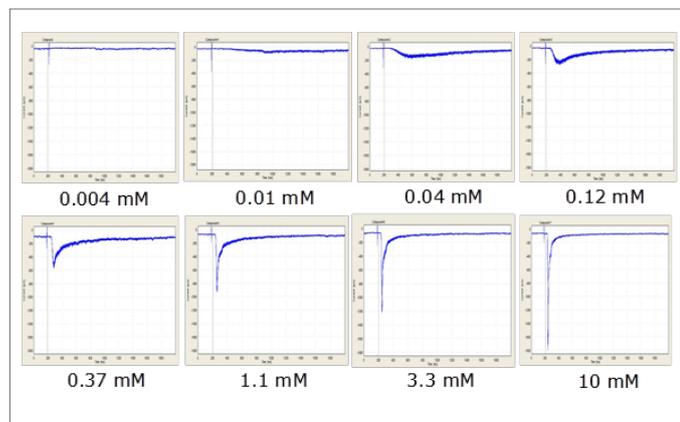


Fig. 4. Raw data traces from a single cell exposed to increasing concentrations of acetylcholine. There was a 150 s break between each addition

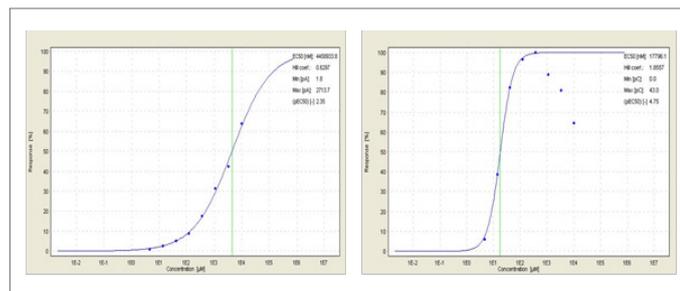


Fig. 5. Left panel: Hill fit plot of the peak currents. Right panel: Area under curve (AUC) analysis of the same data.

The EC<sub>50</sub> was estimated to 2.0 ± 0.7 mM (n=8) using the peak current response. This value is right shifted when compared to data obtained on the Dynaflo system where the EC<sub>50</sub> was found to be 375 µM (1). This discrepancy is most likely due to the fact that the liquid exchange time is limiting for a “correct” estimation of the peak value. However, when employing an area under the curve (AUC) based Hill fit there is very good agreement between the two systems: 26 µM found on QPatch vs. 29 µM on dynaflo. Figure 5 shows the concentration-response plots based on the two methods.

We further tested the response stability using double agonist EC<sub>50</sub> experiments. Two times five-point acetylcholine dose-response experiments were done in sequence on the same cell to evaluate if the responses were stable at a wide range of concentrations and over an extended period of time.

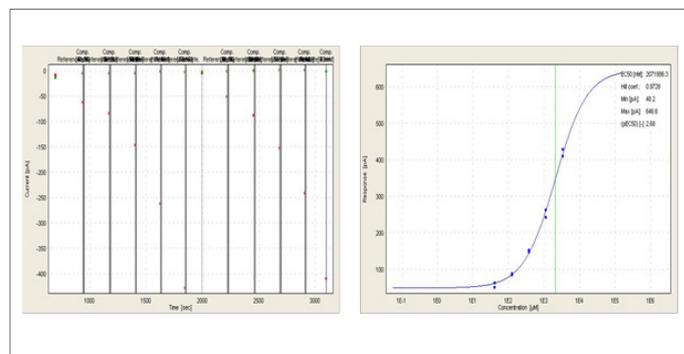


Fig. 6. A shows a typical I/t-plot. These experiments lasted app. 45 minutes. B shows an overlay of two EC<sub>50</sub> curves from the same cell to better illustrate the reproducibility of the responses.

The results are shown in Figure 6 and Table 1. It can be seen that the responses are highly homogeneous throughout the range of concentrations. This furthermore demonstrates a very high reproducibility which is essential for pharmacological studies.

Table 1. Summary of the averaged results from 5 cells.

[Acetylcholine] (µM)	(peak 1 / peak 2)*100 (%) ± SEM
41	101 ± 13
123	105 ± 8
307	107 ± 10
1111	101 ± 11
3333	87 ± 12

## Partial agonism

Nicotine was used to demonstrate a partial agonist assay. Two additions of 300  $\mu\text{M}$  acetylcholine (max response for AUC and  $\text{EC}_{20}$  for peak) was added to be able to determine full and partial agonism. Figure 7 shows raw data from a typical experiment and the analysis is shown in Figure 8.

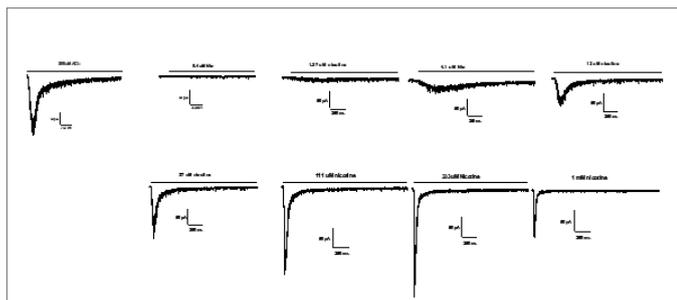


Fig. 7. Raw data plots from a typical partial agonist test assay. Top left sweep is one of the 300  $\mu\text{M}$  ACh control sweeps and the rest are increasing concentrations of nicotine. The highest concentration is 1  $\mu\text{M}$  and the dilution factor is 3.

The AUC  $\text{EC}_{50}$  for nicotine was found to be  $2.8 \pm 0.1 \mu\text{M}$  ( $n=5$ ) and the peak  $\text{EC}_{50}$  was found to be  $20.1 \pm 5.4 \mu\text{M}$  ( $n=5$ ). These results are in very good agreement data obtained using the Dynaflo system (AUC  $\text{EC}_{50} = 9.9 \mu\text{M}$ ).

It can be seen that the typical bell-shaped response curve was also obtained with nicotine using peak current values (figure 8 top right).

It is clearly seen that  $\text{EC}_{20}$  of acetylcholine results in an AUC of about twice the size of the maximal nicotine response (Figure 8 top left). Acetylcholine ( $\text{EC}_{20}$ ) elicited a peak current that was comparable to the maximum nicotine response (333  $\mu\text{M}$ ). Since the maximum response of nicotine only evoked a signal comparable to the  $\text{EC}_{20}$  signal of acetylcholine, one can conclude that nicotine, in fact, is a partial agonist.

The data further suggest that nicotine has a higher affinity but a lower efficacy than acetylcholine.

The results on the QPatch therefore nicely reproduce the Dynaflo data that Wyeth Research has made with these agonists.

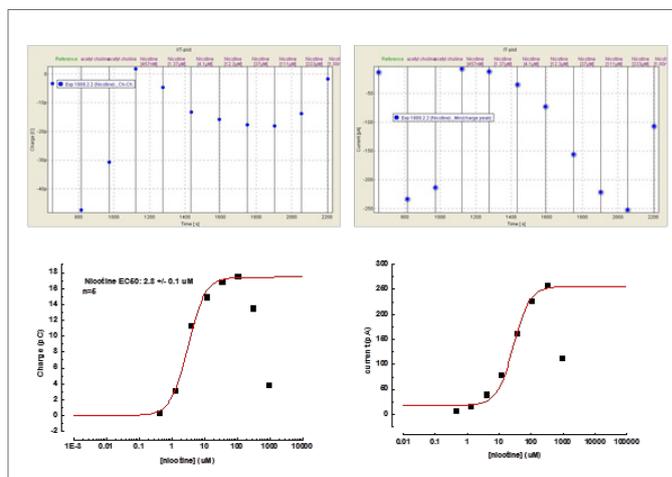


Fig. 8. Left panel top shows AUC/t plot and the respective Hill fit analysis is shown on the bottom. Peak I/t plot is shown in the top right figure and the corresponding hill fit analysis of the same experiment on the bottom.

## Modulator and Antagonist Dose-Response Assays Antagonist Assay

The assay for examining the effect of antagonists and modulators was set up in the same way as the partial agonist assay. A sequence of controls with either 300  $\mu\text{M}$  or 1000  $\mu\text{M}$  acetylcholine were applied before the test compound was added to the cells. The test compound was added alone as a part of the wash from the previous cycle and the cell was thus preincubated for a specified period of time (typically 150 s). Cells were stimulated with acetylcholine plus the test compound following this preincubation step.

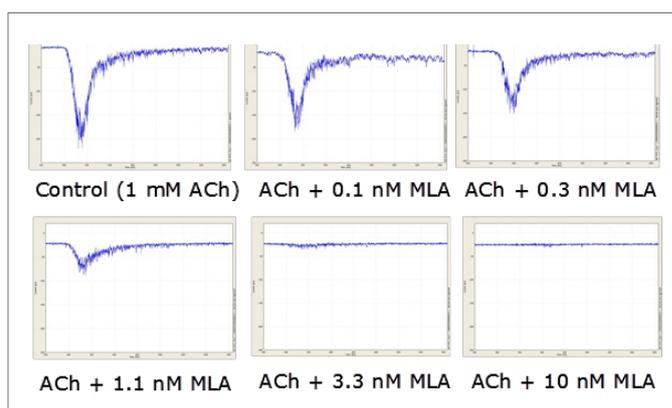
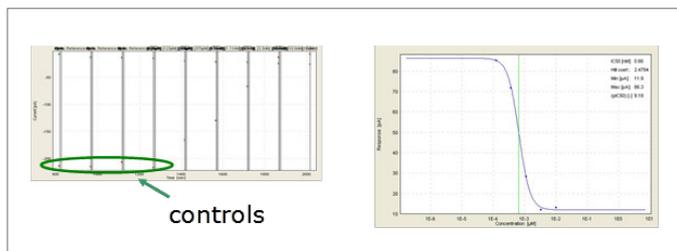


Fig. 9. Raw data traces of responses to increasing concentrations of MLA. Note that the shape of the response does not change - only the size is decreased.

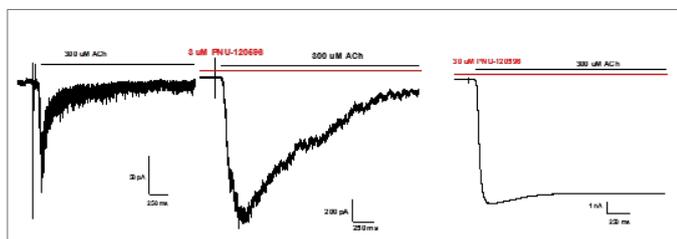
The first compound tested in this way was the highly potent competitive antagonist Methyllycaconitine (MLA). Raw data traces are shown in figure 9 and the analysis is shown in figure 10. The  $IC_{50}$  value was found to be  $0.79 \pm 0.08$  nM (n=9) which is in excellent agreement with published values (2).



**Fig. 10.** Left I/t peak current response plot. The first four additions were acetylcholine controls. Right panel shows the hill fit analysis of the responses. The  $IC_{50}$  was found to be  $0.79 \pm 0.08$  nM (n=9).

### Positive Allosteric Modulators (PAM)

A number of compounds are known to modulate the nicotinic  $\alpha 7$  receptor. The action of these can be divided into two groups: peak current enhancers and gating modulators. These can be discriminated by analyzing both the peak current and AUC.

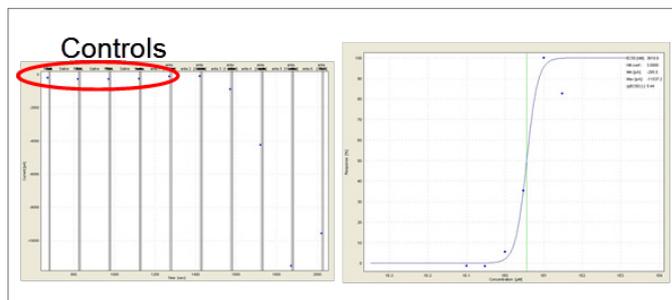


**Fig. 11.** Raw data plots of a PNU-120598 experiment. Left panel is the control middle panel is 3 and right panel is 30  $\mu$ M PNU-120598. Note the scale is changed in each panel.

These groups are not distinct and some compounds can modulate by both mechanisms. Two modulators; one that has a dramatic effect on the desensitization of the channel (PNU-120598) and one with peak enhancing properties (NS1738) were tested on QPatch - both compounds were kindly provided by NeuroSearch.

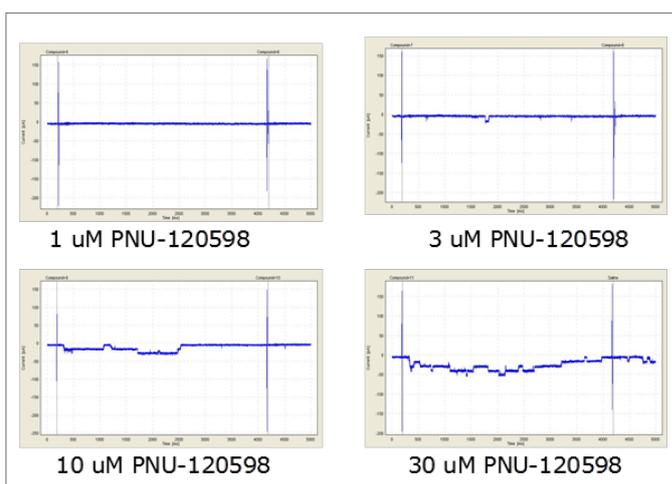
The assay was set up in the same way as the antagonist assay with a number of controls before the test compound was added. In this case, 300  $\mu$ M acetylcholine was used as control followed by preincubation with the test compound for 150 s.

Raw data traces from experiments with PNU-120598 are shown in figure 11. Note the extreme effect on the signal – the desensitization is completely removed by the compound even at 30  $\mu$ M. The  $EC_{50}$  for PNU-120598 was determined to be  $5.5 \pm 1.1$  (n=8). The analysis is shown in Figure 12.



**Fig. 12.** I/t-plot of the peak current response from a typical PNU-120598 experiment. Reliable AUC analysis was not possible due to the extreme effect of the compound.

An interesting observation was made when testing PNU-120598: Some cells that did not exhibit  $\alpha 7$  – mediated current in the initial controls suddenly responded to the increasing doses of PNU-120598 but not with a typical whole cell response. It rather looked like single-channel events that increased in frequency as the concentration of compound was increased. The single-channel conductance seemed to be too high to be  $\alpha 7$  but there was a clear dose-dependence of the responses. A typical example of such a recording is shown in figure 13. These observations indicate that at least part of the cells that did not show  $\alpha 7$ -mediated currents were not true whole cells but rather membrane fragments sitting in the orifice as an outside-out patch. This is probably due to the small size of these cells as described above. It is therefore recommended that an alternative expression system is used for these channels or a size increasing compound like nocodazole is employed.



**Fig. 13.** Single channel events seen in the presence of PNU-120598.

NS1738 is a PAM developed by NeuroSearch that has been shown to increase the peak response to acetylcholine stimulation (3). This compound was also tested on QPatch and the resulting currents can be seen in Figure 14.

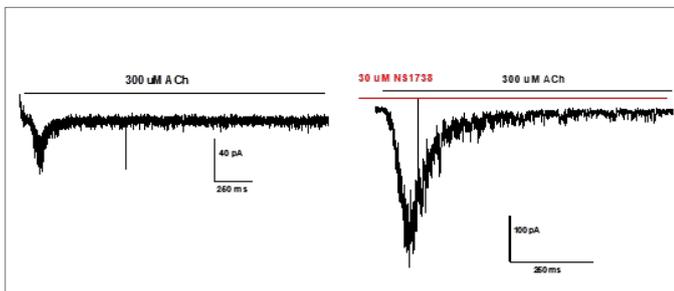


Fig. 13. The modulatory effect of NS1738. This compound mainly increases the peak current.

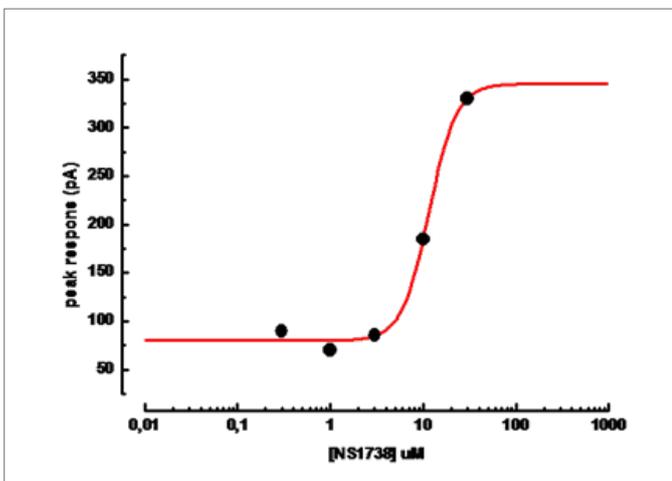


Fig. 14. Hill fit of a 5 point dose response experiment with 300 μM acetylcholine as the test concentration

## Methods

### Cells and electrophysiology

GH4-C1 expressing the rat nAChR  $\alpha 7$  were grown according to Sophion SOP and then used on QPatch. The application of negative pressure applied during seal formation and the pressure pulses used to gain whole cells access were specified in the QPatch assay software, as well as addition of agonists and test compounds were also set up using the QPatch software. During recording, the membrane potential was held at -90 mV.

### Conclusion

In this report, we show that QPatch can be used to measure very fast ligand-gated ion channels in a very reliable manner. The system is able to catch the current in a way that is comparable to a manual set-up and the data produced has a quality that is in very good agreement with the published data.

The platform is both suited for PAM, blocker and agonist characterization and we have here demonstrated that QPatch is capable of handling all of these types of assays.

### References

1. Dunlop et al. In vitro screening strategies for nicotinic receptor ligands, *Biochemical Pharmacology* 74: 1172–1181 (2007)
2. Briggs CA and McKenna DG. Effect of MK-801 at the human  $\alpha 7$  nicotinic acetylcholine receptor. *Neuropharmacology* 35: 407-414 (1996).
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