SOPHION BIOSCIENCE

Application Report

Fast desensitizing ion channels recorded on Qube 384

Short ligand exposure times (<1 s) enable stable recordings of fast desensitizing ion channels, here exemplified by nicotinic acetylcholine receptor alpha1

Summary

- The nicotinic acetylcholine receptor alpha1 (nAChR α1) is a fast desensitizing channel that makes the development of a stable high throughput assay on automated patch clamp systems challenging.
- The solution stacking feature on Qube reduces ligand exposure times below 1 s. Using this feature, it is now possible to evoke stable acetylcholine signals for agonist concentrations at least up to EC_{50}.
- An assay for nAChR α1 endogenously expressed in TE671 cells with 5 µM acetylcholine showed the following characteristics:
  - Overall success rate of 89%
  - Overall whole-cell seal resistance = 1.2 ± 0.4 GΩ (SD)
  - Coefficient of variance for rows and columns in DMSO control ≤12%
  - IC_{50}(max/min)(tetracaine) of a non-cumulative concentration response experiment = 1.5
  - Z' greater than 0.70
- The assay stability is also owed to unique architecture of the QChip where microfluidic channels ensure rapid and complete solution exchange of compound and agonist.

Introduction

Nicotinic acetylcholine receptors (nAChRs) are a diverse family of ion channels that open upon binding with endogenous or exogenous ligands such as acetylcholine or nicotine, respectively. So far, a total of 17 different nAChR subunits (α1–α10, β1–β4, γ, δ and ε) have been described in vertebrates that co-assemble to form a great variety of heteropentamers, with often distinct biophysical and pharmacological characteristics. nAChRs are most prominently expressed in the central and peripheral nervous systems where their main task is to transduce electrical signals (Zouridakis et al. 2009).

The nAChR α1 subunit is predominantly expressed in skeletal muscle cells and it mediates fast agonist transmissions of electrical signals from motor neurons. Following agonist stimulation, the channel becomes non-selectively permeable to cations. Consequently, the membrane potential depolarizes, potentially leading to an excitatory postsynaptic potential in neurons (Kalamida et al., 2007).

Prolonged or repeated agonist exposure to the channel leads to a decreased responsiveness over time, a process called desensitization (Quick & Lester, 2002). Whilst this process is of great physiological relevance, it poses a challenge when studying compound effects on the channel in an *in vitro* setting. In an ideal assay, the exact same current response is evoked following each repeated agonist stimulation. In this way any possible change in current can be attributed to the compound tested.

All 384-well automated patch clamp systems have a single pipetting head. Once the agonist - containing solution is applied to the chip, the robot must clean the pipette tips, load the wash solution and then dispense it to the chip. During the time course of this process, some ligand-gated channels have already desensitized. In the case of nAChRs, addition of acetylcholine esterase to the wash solution helps minimizing this signal decrease over time, however, the esterase is expensive and therefore not an ideal solution to the problem. We recently introduced a novel feature to Qube that allows the stacking of two solutions on top of each other in the same pipette. Using this technique, it is now possible to reduce the ligand exposure time below 1 s. In this present study we developed a stable assay for the relatively fast desensitizing nAChR α1 using the solution stacking feature.

Results and discussion

A 384-well high throughput assay was developed using the solution stacking feature that enables the screening for modulators of nAChR α1. In a first step, the assay was characterized with regard to acetylcholine sensitivity and biophysical properties. At a holding potential of -90 mV, the application of 5 µM acetylcholine evoked an inward current with fast opening kinetics that slowly deactivated over time. A wash step with saline completely abolished this current. As illustrated in figure 1, the agonist exposure time was below 1 s.

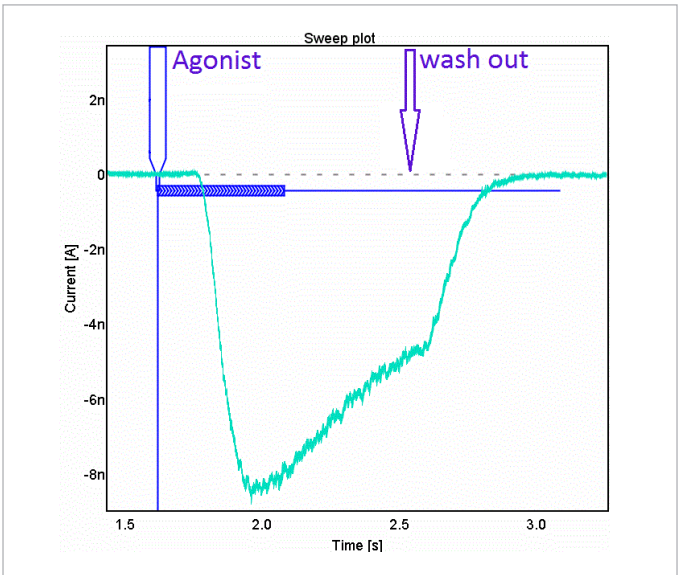

Fig. 1: Typical acetylcholine (5 µM) - evoked current at V = -90 mV in TE671 cells recorded using multi hole QChips. The wash solution (saline) was stacked in the pipette on top of the agonist solution, in this way the agonist could immediately be washed out following application. The flow channel architecture of the QChip allows for complete removal of the agonist, a fact that further reduces desensitization of the channel.

A non-cumulative concentration response curve (CRC) with 8 concentrations of acetylcholine was recorded to determine the half maximal effective concentration (EC_{50}) (Figure 2). Acetylcholine concentrations greater than 1 µM caused an opening of the channel and the full response was reached at concentrations above 100 µM. The calculated EC_{50} value was 9.5 µM, which is in good agreement with results obtained on QPatch (8.5 µM) and values reported in the literature (7.8 µM (Shao et al., 1998)). The good alignment of EC_{50} values between the different techniques provides strong evidence that the agonist solution does not mix with the wash solution in the pipette during the solution stacking step. A mixing of the two solutions would decrease the applied concentration of acetylcholine and hence right shift the resulting EC_{50} value.

A further characteristic property of nAChR α1 is its concentration-dependent gating kinetics. The Analyzer software allows to easily extract advanced biophysical parameters, such as the time between minimum and maximum of a given current response (rise time). The typical concentration-dependent gating of the channel is illustrated by plotting the rise time from 10-90% of the response against the applied agonist concentration (right panel of figure 2). Exposure to 100 µM acetylcholine caused a very rapid opening of all channels with a rise time of 64 ± 6 ms. Fast and complete solution exchange is integral when investigating fast ligand-gated ion channels. The capability of the Qube to detect such fast rise times is owed to the unique architecture of the QChip where microfluidic channels ensure rapid and complete solution exchange.

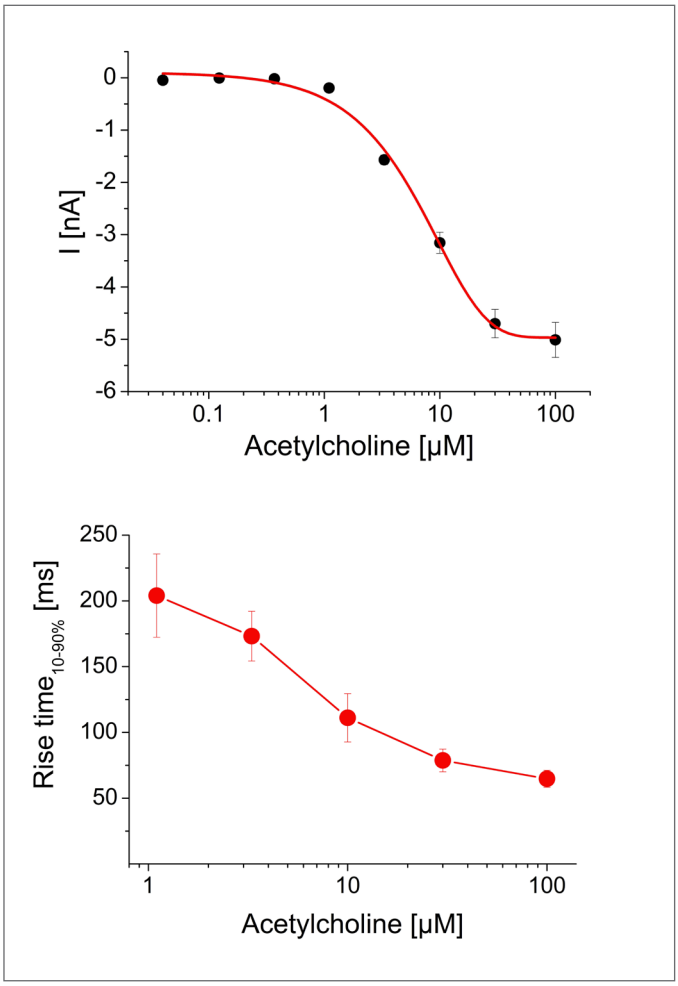

Fig. 2: Non-cumulative concentration response curve (CRC) for acetylcholine. Top: A Hill equation was fitted to the data and the calculated EC_{50} value was 9.5 µM. This value is in good agreement with values measured on QPatch (8.1 µM) and values reported in the literature (7.8 µM (Shao et al., 1998)). Bottom: Time from 10 – 90% of maximum current amplitude at different concentrations of acetylcholine. All data are represented as mean ± SD of n = 39 – 48 experiments per concentration.

In the second step, stability and plate uniformity of the assay was tested. Each stacked additions of 5 µM acetylcholine (EC_{40}) and saline solution was followed by an additional wash step with saline. The second acetylcholine stimulation was used as baseline. Following the baseline response, cells were pre-incubated with the test reagent and compound effects were assessed by applying acetylcholine (5 µM) in the presence of the test reagent (figure 3 upper panel). Inhibition of the acetylcholine-evoked current was calculated as relative to the baseline response. In contrast, using the standard block for ligand-gated ion channels, it was not possible to elicit a second acetylcholine response (figure 3 lower panel). The standard method requires the pipettes to be flushed after agonist application, and wash solution to be taken up, which results in a ligand exposure time of about 30 s.

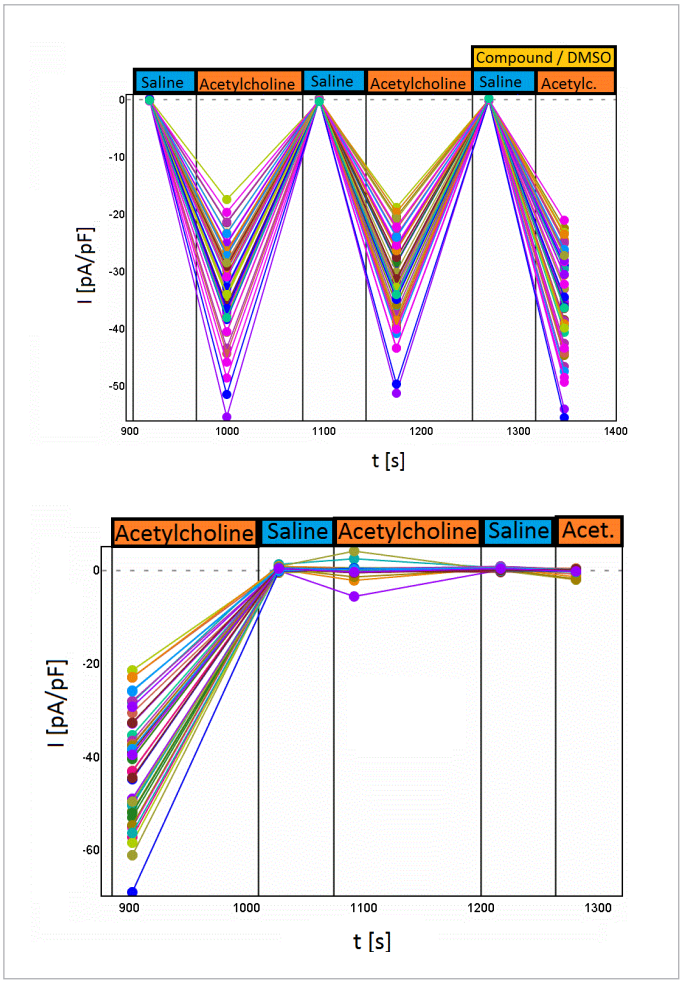

Fig. 3: Current over time (It) plot for the DMSO (0.1%) control. Each stacked application of 5 µM acetylcholine was followed by an additional wash step with saline. No acetylcholine esterase was used in the shown experiments. Upper panel: Two agonist stimulations were used to gain a stable baseline followed by a preincubation step with the respective test compound. Compound effects were assessed in the last step by applying a solution containing both acetylcholine and the test reagent (here 0.1% DMSO). Lower panel: Same protocol as above but using the standard ligand-gated method instead.

To test the assay stability, cells were exposed to the following:
Column 1-23: negative control (0.1% DMSO)
Column 24: positive control (30 µM tetracaine)

Current inhibition values of this experiment are summarized in figure 4. Mean ± 3 times SD are indicated by a bold and two dotted blue lines, respectively. The mean inhibition of DMSO control was -12 ± 10%. Column 23 and column 24 were used to calculate Z' = 0.72. The Z value was calculated from column 1-23 and column 24 and was 0.71.

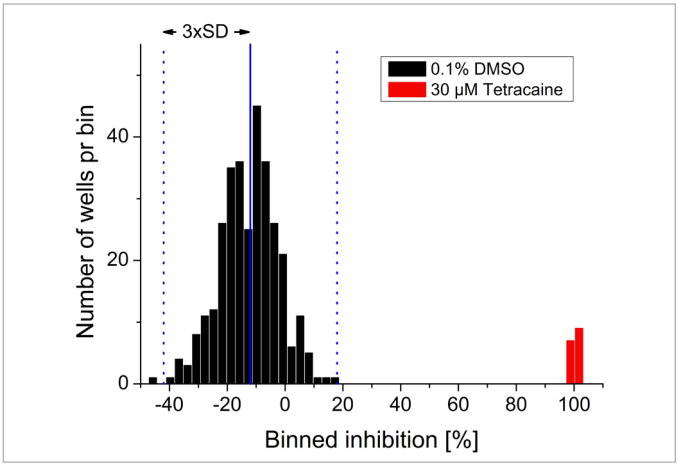

Fig. 4: Histogram plot of DMSO control % inhibition data (black) and 30 µM tetracaine (red). The mean % inhibition of DMSO control is shown as a bold line and 3 x SD (30%) are indicated with dotted lines. Only a single false positive was detected in the shown assay (inhibition > 3 X SD). Z' was calculated from column 23 (negative control) and column 24 (positive control) and was 0.72. The Z value was calculated from column 1-23 (negative control) and column 24 and was 0.71.

Next, the inhibition values were used to assess plate uniformity. Coefficients of variance (CV) were calculated for all rows and columns and the values are represented in table 1. All CV values were below 13%, (Average 8.7%) indicating low data spread.

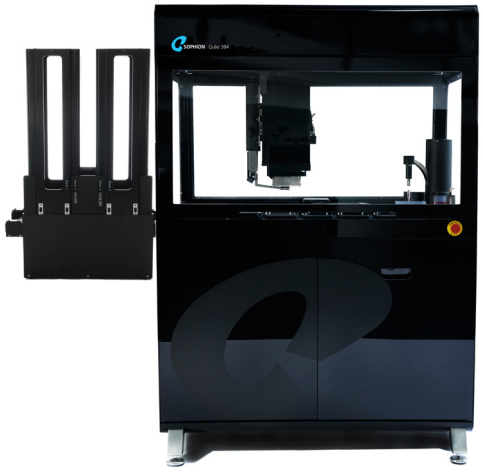

Table 2: Coefficient of variance (CV) calculated from the DMSO control values shown in Figure 4

Group name	CV [%]	Group name	CV [%]
Group DMSO; Row 1	5.9	Group DMSO; Col 22	4.4
Group DMSO; Row 7	5.9	Group DMSO; Col 14	5.8
Group DMSO; Row 12	7.3	Group DMSO; Col 10	6.3
Group DMSO; Row 11	7.8	Group DMSO; Col 11	7.2
Group DMSO; Row 8	7.8	Group DMSO; Col 9	7.3
Group DMSO; Row 9	7.9	Group DMSO; Col 15	7.3
Group DMSO; Row 6	8.2	Group DMSO; Col 8	7.6
Group DMSO; Row 14	8.5	Group DMSO; Col 13	7.6
Group DMSO; Row 4	9.1	Group DMSO; Col 19	7.8
Group DMSO; Row 15	9.3	Group DMSO; Col 1	8.3
Group DMSO; Row 3	9.9	Group DMSO; Col 12	8.4
Group DMSO; Row 5	10.0	Group DMSO; Col 21	8.4
Group DMSO; Row 16	10.6	Group DMSO; Col 23	8.9
Group DMSO; Row 13	10.8	Group DMSO; Col 4	9.0
Group DMSO; Row 10	10.8	Group DMSO; Col 20	9.2
Group DMSO; Row 2	11.1	Group DMSO; Col 18	9.2
		Group DMSO; Col 16	9.9
		Group DMSO; Col 3	10.0
		Group DMSO; Col 17	10.6
		Group DMSO; Col 2	10.7
		Group DMSO; Col 5	11.4
		Group DMSO; Col 6	12.0
		Group DMSO; Col 7	12.3

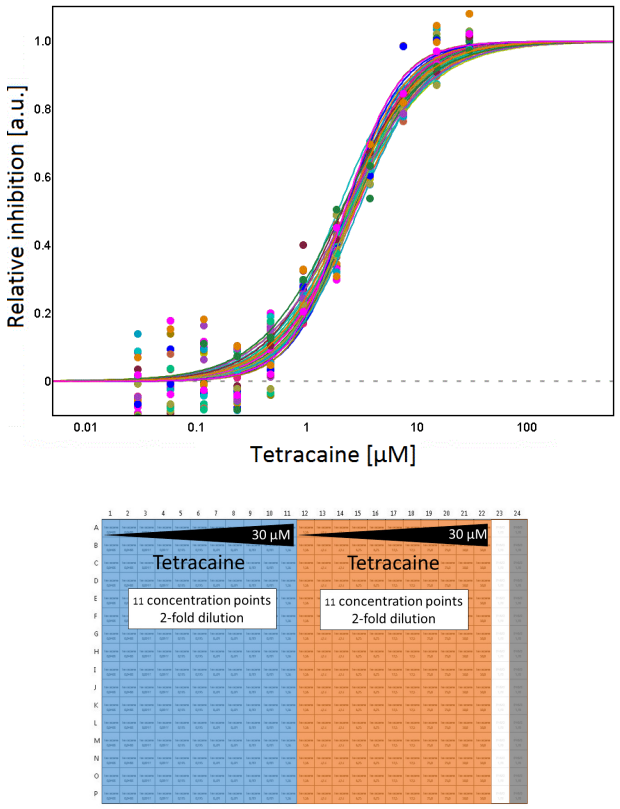

Fig. 5: Non-cumulative CRC of tetracaine. Top: 32 CRCs that were constructed from 11 concentration points with n = 1 per concentration as shown in the bottom insert. The average IC_{50} was 2.4 ± 0.2 µM (SD) and IC_{50}(max/min) = 1.5. This value is in good agreement with a value recorded on QPatch (1.6 µM). Z' was calculated from column 23 (negative control) and column 24 (positive control) and was 0.7.

The assay was pharmacologically characterized using tetracaine, a typical inhibitor of voltage-gated sodium channels that also inhibits nAChRs. An 11 concentration point, non-cumulative CRC was recorded using the compound plate shown in figure 5 (right insert). Each row was used to construct two individual CRCs, each with n=1 per concentration. 32 Hill equations were fitted to the data (figure 5 left panel). The calculated mean IC_{50} value was 2.4 ± 0.2 µM (SD) and IC_{50}(max/min) = 1.5. This value compares well with values recorded on QPatch (IC_{50} = 1.6 µM). Furthermore, the assay showed a very low data spread with Z' = 0.7 (calculated from column 23 (negative control) and column 24 (positive control)).

In conclusion, we developed a stable assay for the nAChR α1 receptor that showed low data spread and a high degree of reproducibility. The nAChR α1, and many other ligand-gated ion channels, require short ligand exposure times to avoid channel desensitization. In the presented assay, we made use of the solution stacking feature to keep agonist exposure below 1 s. This feature opens up new possibilities for the investigation of fast desensitizing ligand–gated ion channels.

Methods

Cells

- TE671 cells endogenously expressing the nicotinic acetylcholine receptor alpha1 subunit (nAChR α1) were cultured at 37°C and 5% CO2 in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin and Streptomycin, and 2 mM sodium-pyruvate. Cells were purchased at ATCC.

  On the day of the experiment, cells were harvested using Detachin (Gelantis) and kept in serum-free media until further use. Qube's automated cell preparation unit was used to resuspend cells in saline just before the start of the experiment.

Electrophysiology

- Whole cell access was gained using a single pressure pulse to -250 mbar for 4 s. Cells were afterwards clamped at Vhold = -90 mV.

- All experiments were performed using multi-hole QChips (10 holes / well).

- The data was analyzed using Sophion Analyzer and Origin 7.5 (OriginLab) software. All data are represented as mean ± SD.

References:

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