Adaptive voltage control ensures the precise half inactivation application of voltage gated channels on automated patch clamp system

全自動パッチクランプシステムによるアダプティブコントロールを用いた正確な50%不活性化状態制御実験の実現

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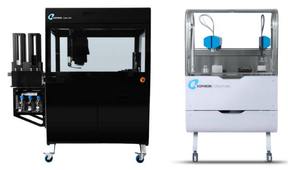

Introduction

Voltage-gated sodium channels have been being studied extensively due to their potentials as targets for several indications such as pain, epilepsy, cardiac and muscle paralysis. Many of the compounds modulating these channels are state-dependent and preferentially bind to the inactivated state of the channel[1,2,3]. The new QPatch II is equipped with the newly developed capability to run online adaptive protocols which makes it possible to measure a half-inactivation potential (V½) in each individual cell and this value may subsequently be used in e.g. a preconditioning pulse in the following voltage protocols . Using this new adaptive protocol feature we determined IC_{50} values for both the closed and the inactivated states for a set of standard compounds. Here we show that the use of individual V½ reduces data variability compared to traditional standard methods.

Materials and methods

Cell preparation: Cells expressing sodium channel $Na_V1.5$, $Na_V1.7$ or $Na_V1.8$ were cultured according to the Sophion SOP. HEK293 cells heterogeneously expressing $Na_V1.5$ were obtained from CreaCell. CHO cells heterogeneously expressing $Na_V1.7$ or $Na_V1.8$ were obtained from Anaxon or Charles River Laboratories (Chantest), respectively. The cells were harvested using detachin ($Na_V1.5$) or Trypsin ($Na_V1.7$, $Na_V1.8$) and transferred to serum free medium (EX-CELL® ACF CHO Medium, Sigma-Aldrich, Brøndby, DK) supplemented with 25 mM HEPES, 40 µg/ml trypsin inhibitor and P/S. The cells were automatically washed and resuspended in extracellular buffer using the cell preparation unit of QPatch II or Qube.

Extracellular solution: 145 NaCl, 4 KCl, 1 MgCl2, 2 $CaCl_2$, 10 HEPES, 10 Glucose (mM), 305 mOsm, pH7.4; Intracellular solution: 10 NaCl, 135 CsF, 1/5 EGTA/CsOH, 10 HEPES (mM), 320 mOsm, pH7.3.

Whole-cell patch clamp recording : Whole-cell configuration was achieved using automated patch clamp systems, QPatch II or Qube. QPatch II is 48 channel high quality whole cell patch clamp system and Qube is high throughput screening system with 384 channel. Voltage gated sodium currents were recorded in voltage clamp mode with giga-seal base whole cell patch clamp recording. All data were stored in the database and analyzed using Sophion Analyzer v6.4 software.

Voltage protocol: [NaV1.5] The cells were held at a holding potential of -90 mV for the resting state. The half inactivation voltage ($V_{1/2}$) was measured by voltage step pulses from -100 mV to 0 mV(ΔV = +5 mV) for 4 seconds followed by a test pulse at -15 mV for 50 ms (Fig. 1A). The $V_{1/2}$ was applied for 4 seconds between two stimulation pulses that consisted of a 40 ms depolarization step at -15 mV followed by a 200 ms pulse at 40 mV and a ramp voltage from 40 mV to -90 mV in 110 ms (Fig. 1B). The state-dependent inactivated currents were measured at the second stimulation pulse. [$Na_V1.7$, $Na_V1.8$] The cells were held at a holding potential of -100 mV for the resting state. The half inactivation voltage ($V_{1/2}$) was measured by voltage step pulses from -100 mV to 30 mV(ΔV = +10 mV) for 500 ms followed by a test pulse at 0mV for 20 ms. The $V_{1/2}$ was applied for 500 ms between two stimulation pulses that consisted of a 20 ms depolarization step at 0 mV. The state-dependent inactivated currents were measured at the second stimulation pulse.

Compound application: The test compounds were applied after the current measurement in extracellular saline (EC) application. The inactivation ratio was calculated and compared between the EC and compound applications. The following compounds were tested in this study; [$Na_V1.5$] Amitriptyline, Mexiletine, Phenytoin, Propafenone, Quinidine, Tetracaine [$Na_V1.7$, $Na_V1.8$] Flecainide, Lidocaine, Tetracaine.

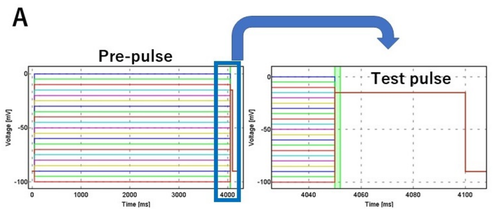

Figure 1. Voltage protocols used for $Na_V1.5$ study
A) Voltage protocol used to measure the half inactivation potential. The voltage steps around the test pulse is enlarged. B) Voltage protocol used to elicit the currents by the first pulse (P1) and second pulse (P2). The cells were clamped at $V_{1/2}$ potential for 4 seconds prior to the P2.

$V_{1/2}$ measurement using adaptive protocol on QPatch II

The half inactivation potentials ($V_{1/2}$) were determined by two ways: online analysis with adaptive protocol during the experiment and offline analysis after the experiment. Figure 2 shows the result of $V_{1/2}$ values calculated in those two different methods. Each cell had different $V_{1/2}$ values calculated by the standard offline method. The $V_{1/2}$ values estimated by the adaptive protocol were very similar values to the ones obtained with standard method with only 0.19 mV difference in average. The result confirms that the adaptive protocol can estimate the $V_{1/2}$ values for each cell in a very accurate manner. Figure 3 shows the typical current sweeps used for the inactivation $V_{1/2}$ estimation and resulting inactivation curve fitted by Boltzmann fitting.

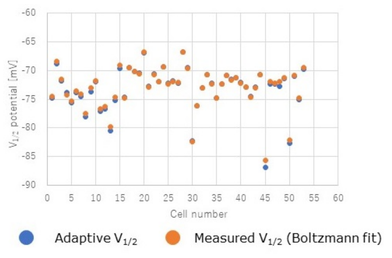

Figure 2. $V_{1/2}$ values measured by two methods
The $V_{1/2}$ values measured by online adaptive protocol (orange) and offline analysis (blue) from multi-voltage steps. The average $V_{1/2}$ values were -73.1±0.5 mV(mean±S.E., n=53) and -73.0±0.5 mV measured by offline and online analysis, respectively. The average difference between online and offline measurements was 0.19±0.05 mV(mean±S.E.). There was no significant difference (P>0.1, t-test).

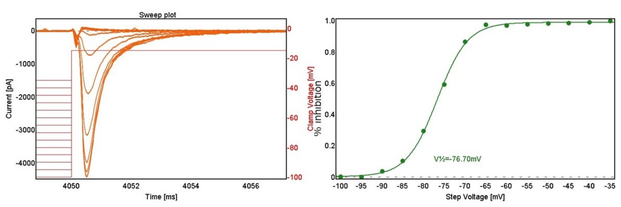

Figure 3. Typical current sweeps and inactivation curve measured by multi-voltage steps
Left: The current sweeps at the test pulses(-15 mV) after applying the prepulse (-100 to 0 mV) for 4 seconds. Right: The $V_{1/2}$ value was estimated by fitting the Boltzmann equation to the data obtained by the multiple voltage steps of pre pulse. The pre-pulses caused the inhibition of the current elicited by the test pulse at higher voltages. The estimated $V_{1/2}$ was -76.7mV in this case.

State-dependent inactivation by applying individual $V_{1/2}$

The individual $V_{1/2}$ was applied to each cell to obtain the half inactivation state, in which half of $Na_V1.5$ channels were in the inactivation state. Ideally, the inactivation currents measured by the second test pulse (P2) decreases in a half size of the first activation pulse (P1) for the resting state activation of $Na_V1.5$ channels. To assess this inhibition ratio, the current amplitude ratio between P2 and P1 (P2/P1) was calculated (Figure 4). Most of cells showed the inhibition rate within 40-60% range. The average inhibition ratio was 46.2±0.7% (mean±S.E., n=53) with individual $V_{1/2}$ prepulse obtained by adaptive protocol. On the other hand, when the prepulse was set at a fixed holding potential at -75 mV (near the average $V_{1/2}$), the average inhibition ratio was 39.3±2.4% (mean±S.E., n=53). As Figure 5 shows, the variability of inhibition ratio is much higher with fixed average $V_{1/2}$ (-75 mV) application compared to the individual $V_{1/2}$ obtained by online analysis in the adaptive protocol (P<0.01).

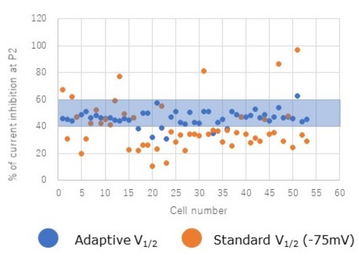

Figure 5. Inactivation ratio by the individual $V_{1/2}$ application vs fixed -75 mV application
The percentage of inhibition currents caused by the prepulse application fixed at the individual $V_{1/2}$ holding potential (blue) or at the holding potential of -75 mV (orange). The current ratio of P2 over P1 (P2/P1) or P2 over maximal current ($P2/I_{max}$) was calculated to show the percentage of inhibition. The blue area shows the range of 50±10%. The individual $V_{1/2}$ application had lower variability compared to the fixed -75 mV application.

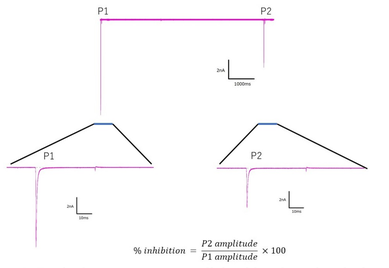

Figure 4. Typical current sweep elicited by the stimulation voltage protocol
The stimulation voltage protocol consists the first pulse (P1) and second pulse (P2) after the 4 second holding voltage at adaptive $V_{1/2}$.

$V_{1/2}$ and inhibition rate using adaptive protocol on Qube

Qube is the higher throughput automated patch clamp screening machine which also equips the adaptive protocol feature. Using Qube, we obtained higher number of data points from over 600 cells. The average $V_{1/2}$ values were -72.3±0.06 mV and -75.1±0.08 mV (mean±S.E., n=699) measured by offline and online analysis, respectively (Figure 6). Most of cells showed the inhibition rate within 40-60% range (Figure 7). The average inhibition ratio was 50.4±0.13% (mean±S.E., n=699) with individual $V_{1/2}$ prepulse obtained by the adaptive protocol. On the other hand, when the prepulse was set at fixed holding potential at -75 mV (near the average $V_{1/2}$), the average inhibition ratio was 36.8±0.28% (mean±S.E., n=699. Same as the results in QPatch II, the variability of inhibition ratio is much higher with the fixed average $V_{1/2}$ (-75 mV) application compared to the individual $V_{1/2}$ obtained by online analysis in the adaptive protocol.

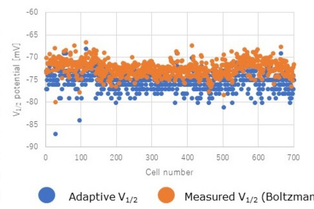

Figure 6. $V_{1/2}$ values measured by two methods on Qube
The $V_{1/2}$ values measured by online adaptive protocol (blue) and offline analysis (orange) from multi-voltage steps. The average $V_{1/2}$ values were -75.1±0.08 mV and -72.3±0.06 mV (mean±S.E., n=699) measured by online or offline analysis, respectively. The average difference between online and offline measurements was 2.8±0.04 mV (mean±S.E.).

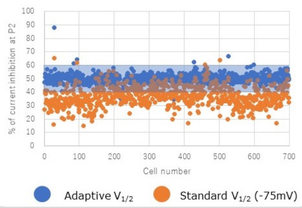

Figure 7. Inactivation ratio by individual $V_{1/2}$ application vs fixed -75mV application on Qube
The percentage of inhibition currents caused by the prepulse application fixed at the individual $V_{1/2}$ holding potential (blue) or at the holding potential of -75 mV (orange). The current ratio of P2 over P1 (P2/P1) or P2 over maximal current ($P2/I_{max}$) was calculated to show the percentage of inhibition. The blue area shows the range of 50±10%. The individual $V_{1/2}$ application had lower variability compared to the fixed -75 mV application (P<0.01, f-test).

Compound effect on the state-dependent inactivation

The compound effects on the both closed state and the inactivated states were evaluated by calculating IC_{50} values and assessment of changes in the inhibition ratio of P2/P1 current. From the IC_{50} values, phenytoin seems to have the highest preferences to the inactivated state to bind to the $Na_V1.5$ channel. However, the P2/P1 inhibition ratio showed the significant decreases at the higher concentrations in all of the compounds tested in this study. This suggests that the P2/P1 inhibition with precise individual $V_{1/2}$ application will contribute to the detection of the compound effect on the inactivated state.

Table 1. IC_{50} values of the selected compounds on the closed state or inactivated state

Group Name	IC_{50} closed [µM]	Hill	IC_{50} inactivated [µM]	Hill	n	IC_{50} closed/inactivated
Group Amitriptyline	13.6	1.3	4.0	1.3	112	3.4
Group Mexiletine	193.7	1.1	31.4	0.9	112	6.2
Group Phenytoin	2192.7	0.5	54.6	0.6	112	40.1
Group Propafenone	6.0	1.1	3.1	1.1	112	1.9
Group Quinidine	92.7	1.0	51.4	1.0	112	1.8
Group Tetracaine	22.4	0.8	3.0	0.9	112	7.5

The IC_{50} values for 6 compounds were measured by application of the compounds at 6 doses for each. The IC_{50} values for closed state and inactivated state were calculated by the inhibition rate of P1 currents and P2 currents, respectively compared to the control period. The dose-response plots were then fitted by Hill equation and the Hill coefficient and IC_{50} values were determined. In the 6 compounds, all of them showed smaller IC_{50} values in the inactivated state, suggesting the preferences to bind the inactivated state for those compounds.

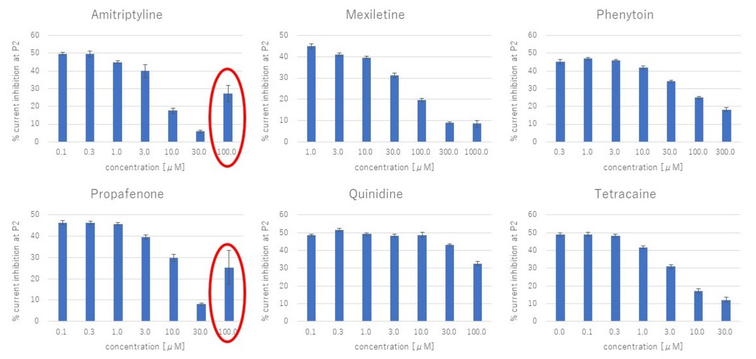

Figure 8. Dose-dependent changes in the inactivation ratio by the compound application (Qube)
The 6 compounds were tested to block $Na_V1.5$ current in the closed state (P1) or the inactivated state (P2). Because the % current inhibition was calculated by dividing P2 by P1 (P2/P1), the current inhibition was not properly calculated when the P1 current was completely blocked by Amitriptyline or Propafenone at the highest concentration (red circles). If the P1 current remains, the P2/P1 ratio could have decreased in a dose-dependent manner. The result suggested that the precise $V_{1/2}$ inactivation state support the accurate detection of the inactivation state-dependent block by these 6 compounds.

Adaptive $V_{1/2}$ application on $Na_V1.7$ and $Na_V1.8$ on Qube

$Na_V1.7$ and $Na_V1.8$, which are known to related to pain sensation, were tested using adaptive $V_{1/2}$ protocol to test the effect of compounds on inactivated state of these ion channels. The adaptive $V_{1/2}$ values were very close to the values of 50% inactivation state potential measured by off-line analysis. Adaptive $V_{1/2}$ value and measured $V_{1/2}$ for $Na_V1.7$ were -57.0±0.19 mV and -56.6±0.07 mV (mean±S.E., n=669), respectively (Figure 9A). Adaptive $V_{1/2}$ value and measured $V_{1/2}$ for $Na_V1.8$ were -33.30±0.11 mV and -34.8±0.10 mV (mean±S.E., n=733), respectively (Figure 9D). The inactivated ratio evaluated by the normalized current size of peak2 and peak1 (P2/P1) was different when the adaptive $V_{1/2}$ was applied for inactivation state compared to standard method (Figure 9 B, E). Adaptive protocol successfully introduced nearly 50% inactivation ratio for individual cells. When the compounds were applied, these compounds shifted the inactivation curve fitted by Boltzmann equation (data not shown). This shift was successfully detected by the changes in P2/P1 ratio of 50% inactivated state introduced by adaptive $V_{1/2}$ application (Figure 9C for $Na_V1.7$ and 9F for $Na_V1.8$). For Lidocaine and Tetracaine, the medium concentration range also gave the sufficient suppression to be detected the inactivation state-dependent effect on $Na_V1.7$ and $Na_V1.8$. Adaptive V1/2 application enables the precise evaluation of the drug effect on inactivated state of those voltage gated sodium channels.

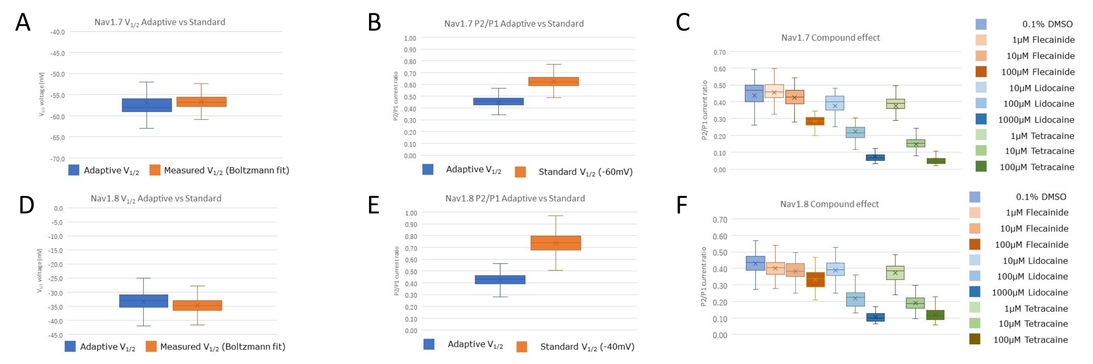

Figure 9. $V_{1/2}$ values of $Na_V1.7$ and $Na_V1.8$ measured by two methods on Qube
The half-inactivated state voltage ($V_{1/2}$) for $Na_V1.7$ (A, B, C) and $Na_V1.8$ (D, E, F) were measured and the adaptive $V_{1/2}$ was applied to each cells to adjust the voltage application to introduce the 50% inactivated state. [A, D] $V_{1/2}$ values measured by adaptive protocol (blue) and off-line Boltzmann fitting (orange) from $Na_V1.7$ (A) and $Na_V1.8$ (D). [B, E] Inactivation ratio (P2/P1) introduced by the adaptive $V_{1/2}$ application (blue) vs standard inactivation potential (-60mV for $Na_V1.7$ (B); -40mV for $Na_V1.8$ (E)) application. [C, F] Changes in P2/P1 current ratio by application of Flecainide, Lidocaine or Tetracaine in three concentrations for each compound on $Na_V1.7$ (C) or $Na_V1.8$ (F).

References

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Summary

Our results demonstrate the feasibility to conduct electrophysiological characterizations and screenings of drug candidates that affect the ion channels in a state-dependent manner by using the adaptive voltage protocol. This new feature enables the automated patch clamp system to apply more accurate individual half inactivation/activation potentials ($V_{1/2}$) during the stimulation phase after the real time online measurement of $V_{1/2}$. With this accurate stimulation, the standard commercialized drugs with state-dependent effects were successfully detected by the $V_{1/2}$ stimulation protocol. Seeking the drugs with state-dependent effect can be done more efficiently by using the new adaptive voltage protocol.