APPLICATION NOTE

Optimization of $Na_V1.5$ channel assay with FLIPR Membrane Potential Assay Kits

Introduction

Voltage-gated ion channels are present in the excitable cell membranes of heart, skeletal muscle, brain and nerve cells. Blocking or modulating such channels can have a therapeutic effect, or may interfere with normal cell function. As a result, compounds that affect voltage-gated ion channels are important targets in drug discovery. Cardiac $Na_V1.5$ channels are classified as "Tetrodotoxin (TTX)-resistant."[1] The pharmacological significance of the $Na_V1.5$ channel is that they are targets for the action of antiarrhythmic drugs and are also blocked by local anesthetics such as lidocaine.[2]

State- and use-dependence

$Na_V1.5$ channels exhibit state- and use dependent inhibition by some compounds such as lidocaine. State-dependent inhibition means that inhibitors bind more readily to a channel when it is in a particular voltage-dependent conformational state (e.g., closed, open, inactivated). Use-dependent inhibition refers to an accumulation of inhibition by a particular compound with repetitive stimuli such as a train of voltage pulses. The pulse train causes the channel to cycle through the voltage-dependent conformational states thus giving the use-dependent compound more cumulative access to its binding site.[3] State- and use-dependence phenomena result in a shift of IC_{50} concentrations of some compounds between membrane potential assays and patch clamp data.

FLIPR® Membrane Potential (FMP) Assay Kits provide a rapid and reliable fluorescence-based method to detect changes in membrane potential brought about by compounds that modulate or block voltage-gated ion channels. It has been previously shown that interaction between test compounds and membrane potential dyes may cause changes in fluorescence response leading to altered estimation of compound activity.[4] Molecular Devices has developed two different no-wash formulations of FLIPR Membrane Potential Assay Kits: Blue and Red. Each kit uses a proprietary indicator dye, combined with different quencher to maximize cell line/channel/compound applicability while eliminating causes of variability in the data. Depending on the chemical properties of compounds in a library, differences in compound auto-fluorescence, or effect of dye on cell type or receptor, one kit may provide better response than the other one.

During assay development, optimal compound response can be empirically determined by testing both kits with a representative sample of the library. Cardiac $Na_V1.5$ channel is used to demonstrate differences in membrane potential assay functionality showing optimization results with both the FLIPR Membrane Potential Red and Blue Assay Kits on the FLIPR Tetra® System.

Materials

- Cell line: Voltage-gated sodium channel $Na_V1.5$ stably expressed in CHL (Chinese hamster lung cells)
- Culture Media: Dulbecco's Modified Eagle's Media (Invitrogen Cat. #11995), 5% Fetal Bovine Serum (FBS, Hyclone Cat #SH30071.03), 1% Pen/Strep/Glutamine (Invitrogen Cat. #103786), 450 µg/mL Geneticin (Invitrogen Cat. #10131-035)

Benefits

- **Detect changes in membrane potential brought about by compounds that modulate or block voltage-gated ion channels**
- **Maximize cell line/channel/compound applicability while eliminating causes of variability in the data**

- Assay Buffer: 10X HBSS (Invitrogen Cat. #1406-5056) diluted in sterile water for injection (Irvine Scientific Cat. #9309), with 20 mM HEPES (Invitrogen Cat. #15630-080). Adjust pH to 7.4.
- FLIPR Membrane Potential Assay Kits (Molecular Devices, Red Cat. #R8123, Blue Cat. #R8034)
- 384-well black wall, sterile, TC treated, clear bottom plates (Corning Cat. #3072)
- Sodium channel modulators:
  - Veratridine (Sigma Cat. #V-5754)
  - Lidocaine (Sigma Cat. #L-7757)
  - Tetrodotoxin (TTX, Sigma Cat. #T-8024)
- FLIPR Tetra System (Molecular Devices)

Parameter	Setting
Excitation/Emission Wavelength	510–545/565–625 nm
LED Intensity	80%
Camera Gain	50
Exposure Time	0.4 sec.
Read Interval	1 sec.
Dispense Volume	12.5 µL
Dispense Height	20 µL
Dispense Speed	25 µL/sec.
Expel Volume	0.5 µL
Dispense Tip Up Speed	6 mm/sec.

Table 1. FLIPR Tetra System Settings for Membrane Potential Assays (384-well).

Methods

Assay plate preparation

To create 384-well assay plates, $Na_v1.5$ cells are trypsinized, re-suspended in culture media, and plated in 25 µL at 12,500 cells/well in blackwall clear-bottom 384-well plates. Incubate microplates overnight at 37°C in 95% humidity, and 5% CO_2.

FLIPR Membrane Potential assays

Step 1: Prepare dye-loading buffer for 10 plates by completely dissolving contents of one FMP Red Assay bulk-kit reagent vial each with 100 mL assay buffer.

Step 2: Remove cell plates from the incubator and add 25 µL of red dye-loading buffer directly to each well in the plate without removing culture media or washing.

Step 3: Incubate dye-loaded plates for 30 minutes at room temperature prior to assay on the FLIPR Tetra System.

Step 4: Prepare the FLIPR Tetra System using the set up parameters, as listed in Table 1.

Step 5: In polypropylene 384-well plates, prepare dose-response series containing 5X concentrations of veratridine, a voltage-dependent sodium channel opener, and 10X concentrations of sodium channel blockers lidocaine, and TTX.

Step 6: On the FLIPR Tetra System, add veratridine to a $Na_v1.5$ cell plate to open the sodium channel and record change in fluorescence as the cell membranes depolarize. Determine the effective concentration for an 80% maximal response (EC_{80}) for veratridine.

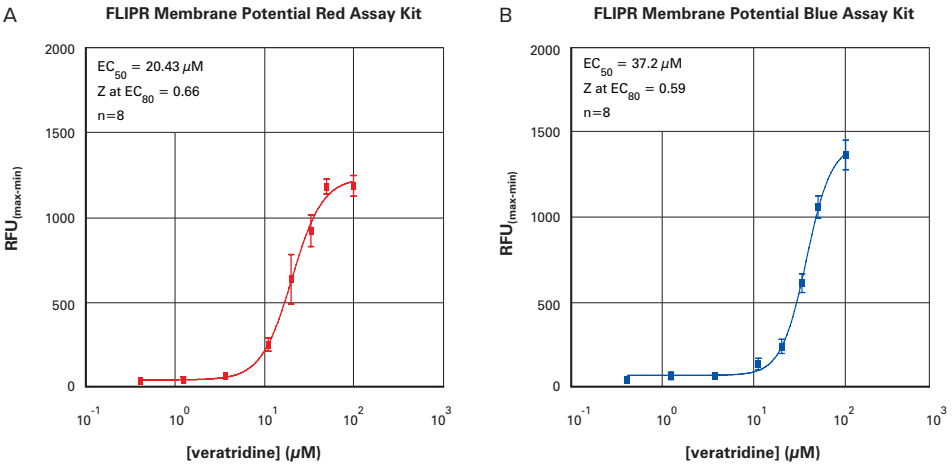

Figure 1. $Na_v1.5$ channel response to veratridine. Comparison of veratridine EC_{50} curves. **(A)** Results from FLIPR Membrane Potential Red Assay Kit. **(B)** Results from FLIPR Membrane Potential Blue Assay Kit showing slight differences in response to veratridine.

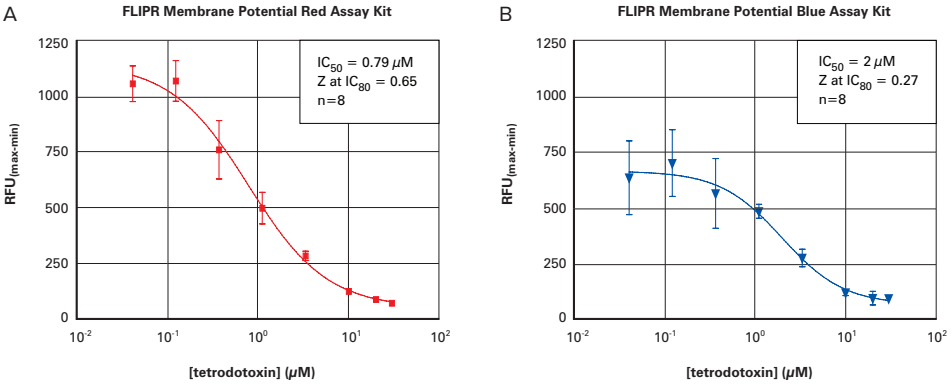

Figure 2. Modulation of $Na_v1.5$ channel in CHL cells by tetrodotoxin. Comparison of tetrodotoxin IC_{50} curves. **(A)** FLIPR Membrane Potential Red Assay Kit. **(B)** FLIPR Membrane Potential Blue Assay Kit. A larger response with a better Z' factor is seen in FLIPR Membrane Potential Red Assay Kit.

Step 7: Offline, add 5 µL 10X channel blocking compounds to additional dye-loaded cell plates and incubate at room temperature for 15 minutes.

Step 8: On the FLIPR Tetra System, add 5X veratridine at EC_{80} concentration and record change in fluorescence during membrane depolarization.

Step 9: Determine IC_{50} concentrations of lidocaine and tetrodotoxin.

Step 10: Repeat assay with FMP Blue Assay Kit.

Step 11: Determine $Na_v1.5$ channel blocker compound IC_{50} concentrations and graph results using Prism GraphPad graphing software.

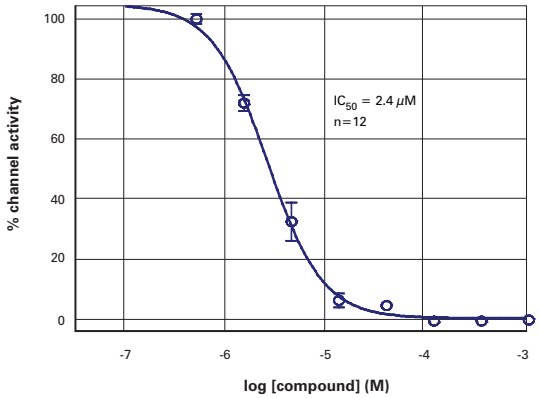

Figure 3. Tetrodotoxin dose-response curve by electrophysiological assay. Dose-response curve for TTX using an electrophysiological assay. An IC_{50} value of 2.4 µM was obtained using an IonWorks HT instrument. The single hole per well PatchPlate™ substrate was used and 12 wells were pooled for each data point. Mean ± SD of the fraction of current remaining after compound addition is plotted.

Results

Veratridine: channel opener

In an electrophysiology assay, voltage is applied to the cell membrane to drive the sodium channel into its open state followed by a rapid movement into the inactivated state. By comparison, in membrane potential fluorescence assays, veratridine is used to hold the sodium channel in its open state preventing inactivation through binding to site two of the six topologically separated toxin binding sites that have been described.[5] Rapid influx of Na^+ into the cell subsequently depolarizes the membrane leading to an increase in fluorescence. As shown in Figure 1, comparison of the cellular response to veratridine using the FMP Red and Blue Assay Kits is similar. EC_{50} concentration using the FMP Blue Assay Kit is slightly right-shifted at 37.2 µM compared to 20.4 µM with the FMP Red Assay Kit.

Tetrodotoxin: channel blocker

Tetrodotoxin is a potent sodium channel blocker isolated from Japanese puffer fish. A 10X dose-response series is added to the cells to block channel opening 15 minutes prior to addition of veratridine. Results showed that FMP Red IC_{50} was 0.79 µM and FMP Blue IC_{50} was 2 µM. Change in fluorescence was greater with FMP Red Assay Kit (Figure 2). In addition, Z factors[6] for FMP Red Assay Kit were higher than those of FMP Blue Assay Kit. A dose-response curve for TTX collected using an electrophysiological assay, generated using the IonWorks® System from Molecular Devices (Figure 3), shows an IC_{50} value

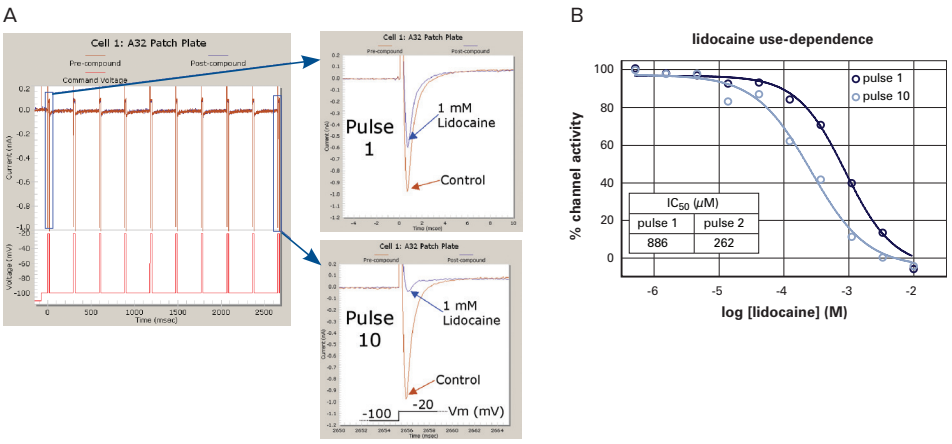

Figure 4. Measuring use dependence. (A) Voltage pulse protocol used to measure use-dependence (left). A ten-pulse protocol is used to elicit currents from a holding potential of -100 mV to the test potential of -20 mV. Raw Na^+ current traces for Pulse 1 (top right) and Pulse 10 of a pulse train voltage protocol. Data was collected using an IonWorks Quattro™ instrument running in Population Patch Clamp™ (PPC) mode. PPC measures the average current from the cells sealed to 64 holes present in each well. Each data point represents the current in a single PPC well showing the fraction of current remaining after compound addition is plotted.

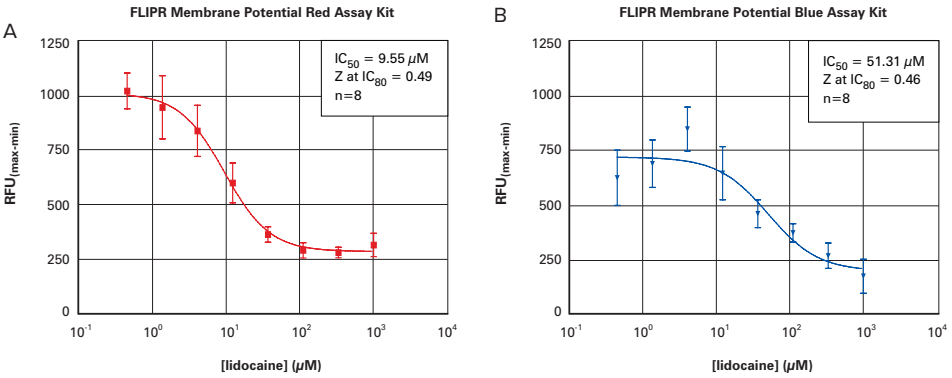

Figure 5. Modulation of $Na_v1.5$ channel in CHL cells by lidocaine. Comparison of lidocaine IC_{50} curves. The FLIPR Membrane Potential Red Assay Kit **(A)** results in a greater fluorescence change and a more sensitive response to lidocaine compared to the FLIPR Membrane Potential Blue Assay Kit **(B)**. Note that, due to use dependence in the $Na_v1.5$ channel, IC_{50} values from the FLIPR Membrane Potential Assay Kits are left-shifted when compared to patch-clamp results shown in Figure 4.

of 2.4 µM. The membrane potential assay results are similar to published conventional patch-clamp data.[1]

Lidocaine: use-dependent channel blocker

Lidocaine is a local anesthetic that works by blocking sodium channels in a use-dependent manner. Use dependent inhibition is seen in IonWorks electrophysiological data in Figure 4, Panel A comparing currents from Pulse 1 to Pulse 10 and showing pulse train and raw Na^+ currents. Dose-response curves for lidocaine are shown in Figure 4, Panel B. IC_{50} values measured at Pulse 1 vs. Pulse 10 are 886 µM and 262 µM, respectively. Evidence of usedependence is present because, as the pulse train progresses from Pulse 1 to Pulse 10, the closed, open, and inactivated states are cycled and the cumulative amount of time that lidocaine is exposed to the open and inactivated state increases. Increased exposure to lidocaine manifests itself as a leftward shift in the measured dose-response curve for Pulse 10.

The FLIPR Membrane Potential Assay Kit results in Figure 5 show that the FMP Red IC_{50} is 9.55 µM and FMP Blue IC_{50} is 51.3 µM. Z factors were higher with the FMP Red Assay Kit than those seen with the FMP Blue Assay Kit. Higher potency measurements in this assay should be expected as veratridine locks the channel in the open conformational state maximizing the exposure of the channel to lidocaine and thus increasing its apparent potency. Follow-up screening by automated patch clamp methods on compounds of interest identified in the FLIPR assay is recommended.

Conclusion

Veratridine can be used instead of voltage to open sodium channels as part of a highthroughput membrane potential screening strategy to identify modulating compounds. As demonstrated by the channel blocker tetrodotoxin and use-dependent channel blocker lidocaine, IC_{50} concentrations can shift, therefore compounds of interest identified on the FLIPR Tetra System should be confirmed using automated or traditional patch clamping methods. With two kit options available, it is possible to optimize a membrane potential assay to provide the best results for specific cell types or compound classes. In this study, both FLIPR Membrane Potential Red and Blue Assay Kits provided good results with veratridine. When used with compounds such as tetrodotoxin and lidocaine, however, the FLIPR Membrane Potential Red Assay Kit gives optimal results.

References

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