

APPLICATION NOTE

Development of a Ca_v1.3 channel assay using optogenetic methods on the FLIPR Tetra System

Introduction

Ca_v1.3 is a L-type voltage-gated calcium channel and an important therapeutic target for drug discovery. It has been shown that a number of drugs exhibit state-dependent effects on Ca_v1.3 channels¹, meaning the potency of these drugs vary in response to the membrane voltage (Vm) and the consequent change of channel states (open, close, inactivated). As this likely provides highly-desired selectivity for pathologically over-activated Ca_v1.3 channels, there is a growing demand for the development of high-throughput assays to evaluate channel blockers under different states.

Current screening methodologies for this channel utilize either electrophysiology or fluorometric methods using potassium challenge to modulate membrane potential, yet both approaches have significant limitations. Electrophysiology directly controls Vm, but it is invasive in nature and not much amenable to high-throughput assays, whereas fluorometric assays bear poor physiological relevance and is not reversible.

To address these limitations, we report here a novel solution using optogenetic methods to control Vm via channelrhodopsin-2 (ChR2).² ChR2 functions as a light-gated non-selective cation channel, primarily permeant to sodium (Na⁺) ions. Upon activation by blue light (470 nm), ChR2 mediates Na⁺ influx which elicits prolonged membrane depolarization driving the Ca_v1.3 channel first to the open state and then over time to the inactivated state. The membrane potential repolarizes following the termination of light, which drives the Ca_v1.3 channel back to its resting state.

In this study, we demonstrate the novel utility of optogenetic tools to control Vm in a reversible and precise fashion for screening state-dependent calcium channel blockers using the FLIPR Tetra[®] System.

Materials

- HEK-293 cell line co-expressing the ChR2_{D156A} (derived from *Chlamydomonas reinhardtii*), human Ca_v1.3 α1 + α2δ, β subunits, and human K_{ir}2.3 was developed by Axxam S.p.A., Milan, Italy. Expression of K_{ir}2.3 sets the Vm initially to a hyperpolarized value, which depolarizes in response to either extracellularly-applied high potassium (acting upon K_{ir}2.3), or blue light stimulation of ChR2 in the presence of all-trans retinal cofactor. In either case, depolarized Vm causes Ca_v1.3 to open, allowing calcium entry into the cell.
- FLIPR Tetra System (0310-5147), FLIPR[®] Calcium 6 Assay Kit (R8190), and FLIPR[®] Membrane Potential Assay Kit (R8042), are all from Molecular Devices, LLC.

Benefits

- Target-specific and non-invasive assay method via light application
- Flexible and reversible assay protocol
- Data quality matching electrophysiology and potassium stimulus

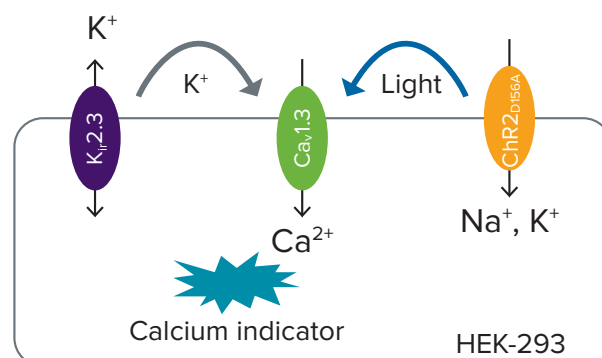


Figure 1. HEK-293 cells transfected with K_{ir}2.3, Ca_v1.3, and ChR2. The influx of calcium ions through Ca_v1.3 is obtained upon depolarization of Vm which can be achieved either with a high K⁺ application, or with light stimulation of ChR2.

Methods

A powerful application of optogenetics is controlling Vm using light-sensitive actuators. In this study, the light-sensitive ChR2 protein from *C. reinhardtii* is used to control Vm as the basis of a high-throughput assay for state-dependent Ca_v1.3 blockers. Specifically, the ChR2_{D156A} mutant is activated by blue light (470 nm) from the LEDs on the FLIPR Tetra System, which induces a robust and prolonged membrane depolarization due to its permeability to Na⁺, and extended time constant for the open state of about 6.9 minutes³. As a result of depolarized Vm, the Ca_v1.3 channel first opens allowing Ca²⁺ ions to enter into the cell, (Figure 1) and over time transits to the non-conductive inactivated state (Figure 2). The kinetic changes in intracellular calcium are monitored using the FLIPR Calcium 6 Assay Kit on the FLIPR Tetra Instrument.

Control of Vm by ChR2

As a first step to validate the control of Vm by ChR2, FLIPR Membrane Potential Dye (Ex 530 nm/Em 565 nm) is used to measure change in fluorescent signal as a result of change in Vm. HEK-293 cells transfected with ChR2, K_v2.3, and Ca_v1.3 are incubated with FLIPR Membrane Potential dye following the standard protocol, in the presence of all-trans retinal co-factor. Using the FLIPR Tetra System, membrane depolarization is induced by stimulating the ChR2 channel with light pulses from the blue LEDs. A second protocol is simultaneously performed which excites the membrane potential dye and measures the change in fluorescent emission over time. The membrane depolarization is reflected by the increase in fluorescent signal. After the termination of the first period of blue light stimulation, ChR2 channels progress to the closed state, allowing Vm to repolarize, resulting in decreased fluorescent signal over time.

Control of Ca_v1.3 by ChR2

HEK-293 cells transfected with ChR2, K_v2.3, and Ca_v1.3 are first loaded with FLIPR Calcium 6 dye, in the presence of all-trans retinal co-factor. Using the FLIPR Tetra System, the cells are pulsed with blue light to stimulate ChR2 and the following change in fluorescence reflects calcium entry into cells through Ca_v1.3. In subsequent experiments, a dual-pulse

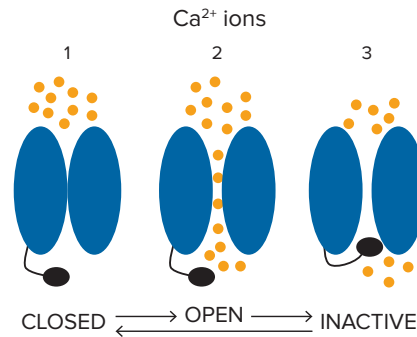


Figure 2. The Ca_v1.3 channel cycles among different states, depending on Vm. When the membrane potential is hyperpolarized, the channel is in its “closed” resting state. Upon Vm depolarization, the channel opens allowing Ca²⁺ ions to enter the cell, then immediately progresses to the “inactivated” refractory state. Upon Vm repolarization, the Ca_v1.3 channel recovers from the inactivation state and returns to the closed state, being then ready to respond again to Vm depolarization stimulus.

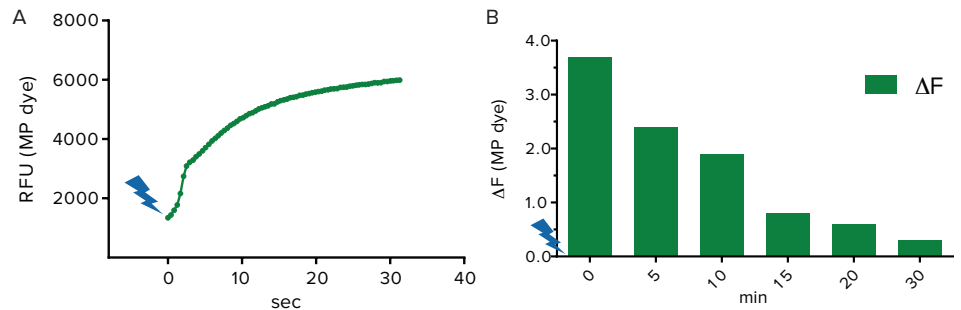


Figure 3. (A) Light-induced depolarization. Depolarization of Vm measured as change in FLIPR Membrane Potential dye fluorescence upon the application of blue light. **(B) Time course experiment showing the change in maximum FLIPR Membrane Potential dye signal over time.** ChR2 Ca_v1.3 HEK-293 cells were incubated with FLIPR Membrane Potential dye. Stimulation of ChR2 by pulses of blue light induced membrane depolarization and resulted in an initial increase in fluorescent signal from the dye (T=0). Following the fluorescent signal over time showed a drop in signal indicating membrane repolarization.

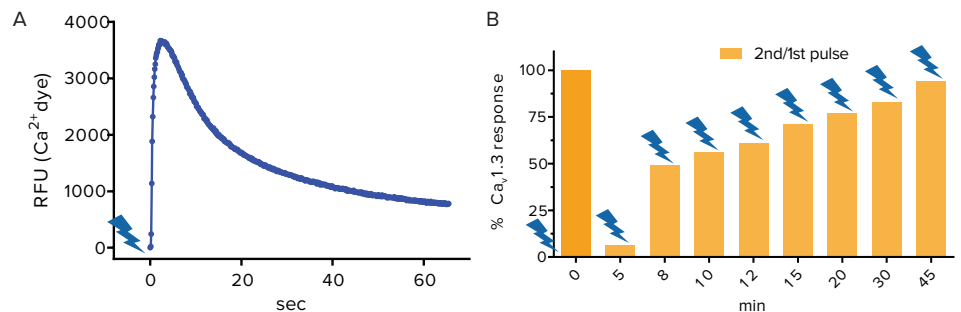


Figure 4. (A) ChR2 induced Ca_v1.3 response after blue light stimulation. The signal trace from the FLIPR Tetra System records the increase in fluorescence of FLIPR Calcium 6 dye as calcium enters the cell through Ca_v1.3. **(B) Ca_v1.3 recovery from the inactivated state.** A dual-pulse protocol was applied here, where the first pulse of blue light was applied at t = 0, then cells received a second pulse after incremental intervals as indicated and calcium flux was measured. Data showing half the initial calcium signal suggests that the half-inactivated state of the channel occurs with the second light pulse delivered 10 minutes after the first stimulation.

protocol was employed, where the second light pulse was applied after varying time intervals after the first pulse to study recovery of $\text{Ca}_v1.3$ from inactivation.

Control of $\text{Ca}_v1.3$ by potassium challenge

As a reference, the standard method of analyzing $\text{Ca}_v1.3$ channel activity by potassium challenge is performed on the same cell line. When high potassium is applied extracellularly, the membrane potential depolarizes allowing the $\text{Ca}_v1.3$ channel to open and calcium ions to enter the cell (Figure 1). Over time, $\text{Ca}_v1.3$ reaches an inactive, non-conductive state. FLIPR Calcium 6 dye is used to measure the increase in intracellular calcium. This method requires the application of high concentration of K^+ to the cells which is non physiological and can have many “off-target” effects. In addition, such application is irreversible as the potassium ions can’t be removed from the well.

Pharmacology

To validate the utility of optogenetics in pharmacological assays, a set of experiments were performed using the state-dependent inhibitor isradipine to block the $\text{Ca}_v1.3$ channel and to analyze IC_{50} results at both the closed and inactivated states. Three methods of activating the $\text{Ca}_v1.3$ channel including optogenetics, high potassium addition based on $\text{K}_v2.3$, and patch clamp are compared to determine isradipine IC_{50} values at both states.

Results

Control of V_m by ChR2

Control of membrane potential by light-activated ChR2 was validated by incubating ChR2 transfected HEK-293 cells with FLIPR Membrane Potential dye. Pulses of blue light from the FLIPR Tetra System LEDs resulted in membrane depolarization and a 3-fold increase in signal from the FLIPR Membrane Potential dye (Figure 3a). Recording the membrane potential signal over time showed that fluorescent signal returned to background levels after about 30 minutes from the initial blue light stimulation as the membrane repolarized. This data suggests that when blue light stimulated ChR2 to open, V_m was depolarized first and over time V_m repolarized as the ChR2 channel closed (Figure 3b), accordingly to its time constant of 6.9 minutes.

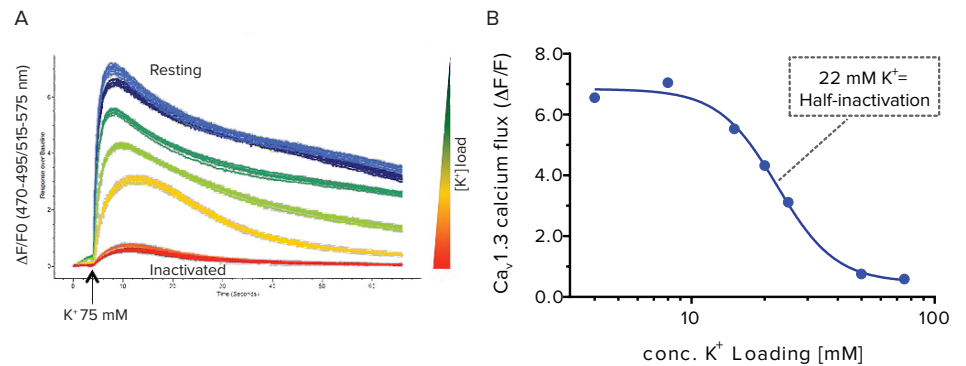


Figure 5. (A) Cells loaded with FLIPR Calcium 6 dye in different $[\text{K}^+]$ to induce different starting voltages. 75 mM K^+ added during detection of calcium signal on FLIPR Tetra System. **(B) K^+ inactivation curve.** By incubating the cells in dye with 22 mM K^+ , 50% of the channels are in the inactivated state.

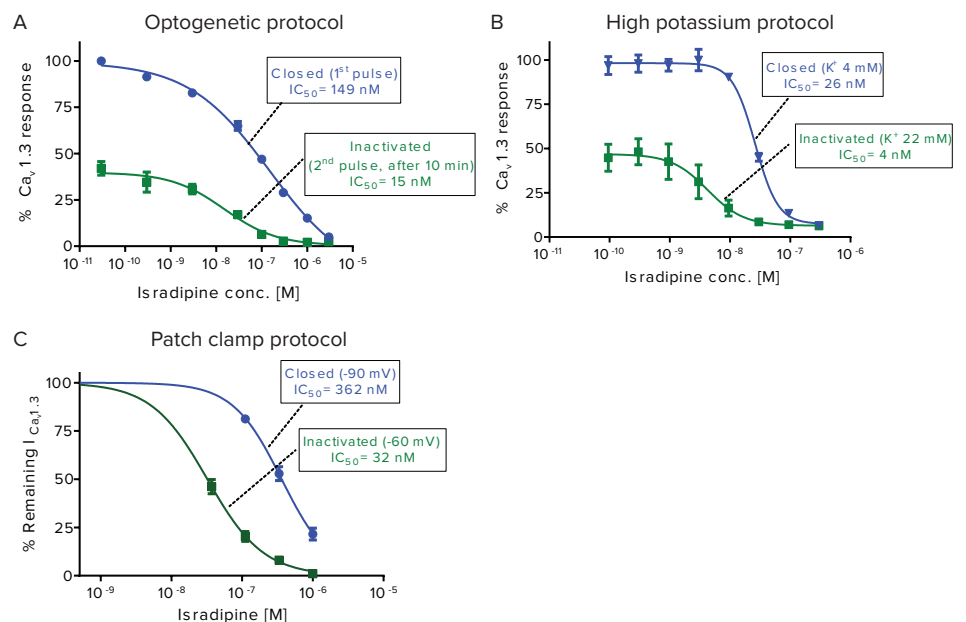


Figure 6. Isradipine state dependency impact upon pharmacology. (A) Optogenetic protocol. At the closed state (first blue light stimulation), isradipine $\text{IC}_{50} = 149$ nM. At the inactivated state (cells re-pulsed with blue light after 10 min), isradipine $\text{IC}_{50} = 15$ nM. **(B) High potassium protocol.** 75 mM K^+ was added during detection. At the $\text{Ca}_v1.3$ closed state (4 mM K^+ load), isradipine $\text{IC}_{50} = 26$ nM. At the inactivated state (22 mM K^+ load), isradipine $\text{IC}_{50} = 4$ nM. **(C) Patch clamp protocol.** At the $\text{Ca}_v1.3$ closed state (-90mV holding potential) the isradipine $\text{IC}_{50} = 362$ nM, and at the inactivated state (-60mV holding potential) the isradipine $\text{IC}_{50} = 32$ nM.

ChR2 induced Ca_v1.3 response

Transfected HEK-293 cells were incubated with FLIPR Calcium 6 dye. Pulses of light from the blue LEDs triggered an increase in fluorescent signal as calcium ions entered the cell (Figure 4a). Subsequent experiments (Figure 4b) in which the cells were subjected to a dual-pulse protocol were used to identify the time-dependence of Ca_v1.3 channel recovery from inactivation. After a 10 min interval, half the calcium flux signal was recorded. After a 45 minute interval, nearly 100% of the signal was recorded. The data suggests that 10 minutes after the first light pulse, half the channels were still in the inactivated state thus resulting in 50% of maximum calcium signal.

Potassium Challenge

To control the Ca_v1.3 channel state with K⁺, the transfected HEK-293 cell line was incubated in FLIPR Calcium 6 dye loading buffer with increasing potassium concentrations from 4 mM to 75 mM for one hour at 37°C resulting in different membrane potentials (Vm). Then a high K⁺ concentration of 75 mM was added during detection of calcium signal on the FLIPR Tetra System. Representative calcium signal responses are shown in Figure 5a. A K⁺ dependent inactivation curve plotting the K⁺ loading concentration vs. the signal from the FLIPR Calcium 6 dye revealed the concentration of K⁺ to induce 50% of maximum Ca²⁺ signal is at 22 mM K⁺ (Figure 5b), suggesting that half the channels were in the inactivated state in this K⁺ loading condition.

Pharmacology

Isradipine, an anti-hypertension drug that may also be useful in slowing the progression of Parkinson's disease, was chosen for this study due to its known state-dependent effect on Ca_v1.3. Using the optogenetic protocol with ChR2,

Isradipine IC ₅₀	Optogenetics	High K ⁺	Patch Clamp	Literature ⁴
Closed State	149 nM	26 nM	362 nM	300 nM (-90 mV)
Inactivated State	15 nM	4 nM	32 nM	30 nM (-50 mV)
RATIO block Closed/Inactivated	9.9	6.5	11.3	10

Table 1. Summary of isradipine IC₅₀ results at both closed and inactivated states. The optogenetic protocol results are the closest to electrophysiological and literature results.

HEK-293 cells expressing Ca_v1.3 were incubated with FLIPR Calcium 6 dye for one hour at 37°C, in the presence of different concentrations of isradipine. Blue light pulses were given on the FLIPR Tetra System and increase in fluorescent signal with calcium influx at the closed state was measured. After 10 minutes, a second pulse of blue light was given recording the change in fluorescence with calcium influx at the inactivated state. Only half of the original calcium signal was seen. Results are shown as % Ca_v1.3 response on the graph in Figure 6a. Isradipine IC₅₀ curves from experiments following the high K⁺ protocol are shown in Figure 6b. Based on earlier potassium experiments, 4 mM K⁺ or 22 mM K⁺ was added to the FLIPR Calcium 6 dye loading buffer to induce closed and inactivated states, respectively. 75 mM K⁺ was added during detection of calcium signal on the FLIPR Tetra System. In Figure 6c, IC₅₀ curves of isradipine block of Ca_v1.3 at both the closed and inactivated states using a patch clamp protocol are shown.

Summary

A comparison of isradipine IC₅₀ values at both closed state and inactivated state including historical patch clamp results is shown in Table 1. An approximately 10-fold left shift in IC₅₀ values at the inactivated state of Ca_v1.3 compared to the values

at the closed state is preserved with all methods. This is important because compound effects upon ion channels can have different clinical implications at closed and inactivated states. With the optogenetic protocol, the IC₅₀ values are closer to the IC₅₀ values obtained with "gold standard" electrophysiology methods. The "ratio block" value obtained with optogenetics is very similar to that obtained with electrophysiology methods suggesting high biorelevance. Unlike electrophysiology assays, the cells in the optogenetics assay are intact at the end of the experiment. In addition, the light-driven optogenetic protocol is reversible, a highly desirable feature for flexible assay protocols.

Acknowledgement

Experimental work and data figures were provided by Axxam S.p.A., Milan, Italy.

Reference

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