Optimizing a transient receptor potential (TRP) channel assay on the FLIPR^{TETRA}® instrument using hTRPV1-HEK293 cells and the FLIPR® Calcium 5 Assay Kit

Carole Crittenden[1], Catherine Parrish[1], Xin Jiang[1], Andy Keys[1], and Steve Smith[2]
[1]Molecular Devices, Inc., Sunnyvale, CA, USA and [2]ChanTest, Cleveland, OH, USA

Introduction

TRPV1 is a nonselective cation channel associated with nociception. This transient receptor family of ion channels can be activated by vanilloids such as capsaicin, elevated temperature or low pH. In the presence of agonist, the pore of the TRPV1 channel is opened in the cell membrane allowing calcium to enter the cell thereby increasing the intracellular calcium concentration. FLIPR® Calcium Kits can be used to indirectly analyze the activity of ligand-gated ion channels by measuring associated changes in intracellular calcium with a fluorescent, cell-based assay. Here we compare performance of the FLIPR Calcium 4 and Calcium 5 Kits to a competitor kit in both agonist and antagonist assays. In addition, EC_{50} values are compared to electrophysiological results obtained on the PatchXpress® 7000A automated parallel patch clamp system. This evaluation demonstrates that the superior performing FLIPR Calcium 5 Assay Kit provides a larger signal window, increased signal to noise ratio, consistent pharmacology, and improved reproducibility.

Materials and Methods

ChanTest™ hTRPV1-HEK293 cells (Cat # CT-HK 011) are a stably transfected cell line with a human TRPV1 channel constitutively expressed in the HEK 293 (human embryonic kidney) cell line. The cells have been validated by ChanTest for use in manual patch-clamp recording, the PatchXpress® 7000A automated parallel patch clamp system, and in conjunction with Calcium Kits on the FLIPR^{TETRA}® system.

Culture Media: DMEM/F12 (Cat# 10-092-CM), 10% FBS (Cat# 35-010-CV) 0.5 mg/mL Geneticin (G418) (Cat# 30-234-CI) all from Mediatech. Cells are subcultured by rinsing the cultures in PBS without Ca2+ and Mg2+ (Cat# 14190-250, Invitrogen) followed by incubation with 1 mL 0.05% Trypsin-EDTA (Cat# 25300054, Invitrogen) for 5 minutes. In preparation for the assay, the cells were plated overnight @ 37 ºC and 5% CO_2 in culture media at 10,000 cells/well in 384-well black, clear bottom poly-d-lysine coated plates (Cat# 356663, BD).

Compounds: capsaicin (Cat# M2028), resiniferatoxin (Cat #R8756), olvanil (Cat# O0257), and capsazepine (Cat# C191) all from Sigma.

Assay Kits: FLIPR Calcium 5 Kit (Cat #R8185), FLIPR Calcium 4 Kit (Cat # R8142), FLIPR Membrane Potential Red Kit (Cat #/R8126), FLIPR Membrane Potential Blue Kit (Cat# R8042), all from Molecular Devices, Inc. and Fluo-4 Direct Kit Cat# F10471, Invitrogen). All calcium and membrane potential assay kits were prepared following the instructions in the package inserts. A 5X volume of CRC or EC_{80} ligand was prepared in HBSS buffer + 20 mM HEPES in 384-well polypropylene plates. Ligand was added during detection on the FLIPR^{TETRA} instrument at optimized parameters. Capsazepine was prepared at 5X concentration and added 15 minutes prior to addition of a 6X volume of EC_{80} concentration of challenge ligand. Relative Fluorescence Units (RFU) were calculated as the fluorescent signal maximum minus signal minimum during 90 seconds after addition. Graphs and EC_{50}/IC_{50} concentrations were calculated using GraphPad Prism® software. Z-factor calculations were performed using the method from Zhang, *et.al.*

Direct Measurement of TRPV1 Currents

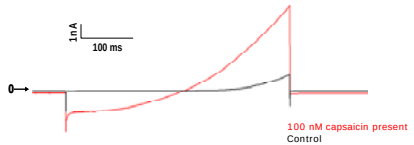

Figure 1. Direct measurements of TRPV1 currents on PatchXpress 7000A Automated Parallel Patch Clamp system. The currents were evoked by a ramp voltage protocol from -100mV to +100mV (Vh = 0mV); in the absence (black line) and presence (red line) of 100 nM capsaicin.

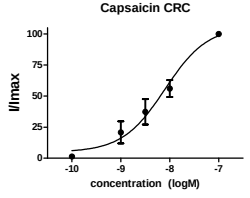

Figure 2. Concentration-dependent activation of TRPV1 channel by capsaicin. For each data point, the peak outward currents (measured at +100mV) were first normalized to the maximum current obtained from the same cell with 100nM capsaicin; and then averaged and plotted (n = 8). Capsaicin EC_{50}= 7.97 nM.

Comparing TRPV1 response using calcium reporter and membrane potential dyes

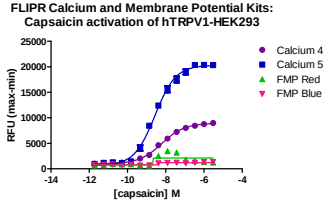

Figure 3. Evaluation of best dye choice for monitoring hTRPV1 activation. Using capsaicin as a model agonist, both calcium reporter and membrane potential dyes were tested. Dyes specific to calcium flux gave the best response.

Measurement of hTRPV1 activation response on the FLIPR^{TETRA} system

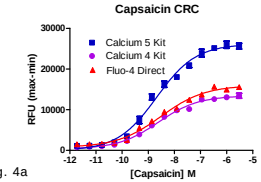

Fig. 4a

Capsaicin	PatchXpress	Calcium 4	Calcium 5	Fluo-4 Direct
EC_{50} (nM)	7.97	2.41	2.10	3.19
Z at EC_{80}		0.64	0.72	0.63

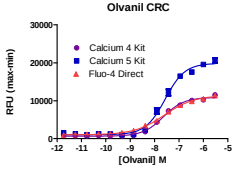

Fig. 4b

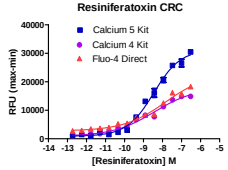

Fig. 4c

	Compound	Calcium 4	Calcium 5	Fluo-4 Direct
EC_{50} (nM)	Olvanil	22.9	27.2	25.2
Z at EC_{80}		0.75	0.84	0.73
EC_{50} (nM)	Resiniferatoxin	3.7	3.4	14.2
Z at EC_{80}		0.70	0.76	0.74

Figure 4. FLIPR® Calcium 5 Kit shows superior performance in calcium mobilization assays of capsaicin challenge to TRPV1 cells. Calcium 5 assay windows approach or exceed two-fold over the Calcium 4 and competitor's kits with improved Z scores and consistent pharmacology. EC_{50} values compare with patch-clamp values seen in Figure 2 as well as published results. Three agonists were used in this study: a) capsaicin, b) olvanil, and c) resiniferatoxin.

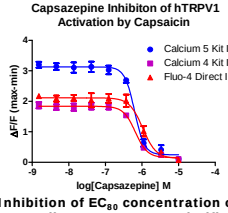

Figure 5. Inhibition of EC_{80} concentration of capsaicin. Results from smaller responses are clarified by the larger signal to background ratio of the FLIPR Calcium 5 Kit.

Discussion

To establish a baseline for results obtained on the FLIPR^{TETRA} system, direct measurements of TRPV1 currents were performed on the PatchXpress 7000A Automated Parallel Patch Clamp System and shown in Figure 1. The concentration-dependent activation of TRPV1 channel by a reference agonist , capsaicin, was analyzed in Figure 2. Assay optimization on the FLIPR^{TETRA} system was continued with the assessment of which fluorescent reporter dye, a calcium flux reporter or general membrane potential reporter, would be the best choice for analyzing activation of the TRPV1 ion channel. As shown in Figure 3, the dyes specific to calcium flux gave the best measureable response to the reference agonist capsaicin. Of the two calcium dyes tested, Calcium 5 Kit outperformed Calcium 4 Kit with a two-fold increase in signal window yet maintained similar pharmacology (EC_{50} = 2.7nm and 6.2nM for Calcium 5 and Calcium 4 Kits, respectively). These results were within range of the EC_{50} value of 7.97nM determined electrophysiologically on the PatchXpress 7000A system.

To test the applicability of Calcium 5 Kit as a superior performing dye choice, we expanded the agonists tested to include two additional commonly used TRPV1 agonists, olvanil and resiniferatoxin. In addition, we compared a competitor's calcium mobilization reporter dye to those that were tested. For each agonist, Calcium 5 consistently resulted in a larger assay window than Calcium 4 or Fluo-4 Direct. Signal increases of up to 100% were seen using the Calcium 5 dye. Finally, capsazepine was used as an inhibitor of the capsaicin response in TRPV1 cells as shown in Figure 5. Calcium 5 Kit again showed an increase in signal to background ratio and more consistent pharmacology over the competitor dye.

Summary

Calcium mobilization reporter dyes are a better choice for monitoring TRPV1 activation than membrane potential dyes.

The EC_{50} value of capsaicin was consistent across both the FLIPR^{TETRA} and the PatchXpress platforms.

For all agonists and antagonists tested, FLIPR Calcium 5 Kit produced a larger signal window (up to a two-fold increase in signal intensity) than FLIPR Calcium 4 or Fluo-4 Direct dye while maintaining pharmacological profile.

FLIPR Calcium 5 provided equal or improved reproducibility for all agonists tested as measured by Z score.

The larger signal window seen with Calcium 5 provides a broader spectrum for antagonist analysis thereby allowing smaller responses to be discriminated.

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