Validation of TRPV1 and ASIC1 ligand-gated ion channels using automated patch clamp and FLIPR with novel Ca^{2+} detection dye

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Introduction

Ligand gated ion channels (LGICs) are a large family of membrane embedded proteins that enable the passage of ions across membranes, in response to the binding of ligands such as neurotransmitters. LGICs represent a class of highly attractive drug targets because of the pivotal role they play in many physiological functions, and their association with multiple human diseases. Among existing ion channel screening technologies, the fluorescent probes, especially the calcium sensing dye, have become indispensable tools for analyzing ion channel activities. The simplicity of the assay, the high throughput, as well as the low running cost, makes this method especially attractive to large scale primary screening in the early phase of the drug discovery process. In an attempt to further improve such assay quality we evaluated the use of a novel calcium-sensing dye in LGIC assays. The unique molecular configuration of this dye, combined with a novel quenching technology, provides distinctive advantages over existing calcium probes by 1) providing a larger signal/noise ratio, and 2) removing the need of probenecid in the assay. Specifically, in this study we examined two calcium permeable ligand gated ion channels: acid sensing ion channel 1a (ASIC1a) and transient receptor potential vanilloid 1 (TRPV1).

CHO-K1 cells stably expressing pore-forming subunit of human ASIC1a channel and HEK293 cells stably expressing pore-forming subunit of human TRPV1 channel were used in the study.

In one series of experiments, we constructed and validated cell based assays in which the FLIPR® Calcium 6 assay kit containing this novel calcium fluorophore was used to examine the activity of ion channels, as well as multiple channel modulators, by measuring changes of fluorescent signals associated with intracellular calcium. The results were then compared to those obtained with a direct electrophysiological method, using the IonWorks® Barracuda Automated Patch Clamp System. Another series of experiments was devised to examine the calcium selectivity of this fluorescent calcium probe. After removing calcium from the external solution, the fluorescent signals became absent upon channel activation; whereas the electrical currents maintained. In conclusion, we demonstrated the sensitivity and selectivity of a novel fluorescent calcium probe, and its successful applications in high-throughput LGIC assays. The combination of this method with automated electrophysiology offers a high throughput, robust platform for LGIC screening in drug discovery.

Methods

Cells:

ChanTest™ hASIC1a-CHO cells (Cat # CT6012) are a stably transfected cell line with a human ASIC1a channel constitutively expressed in the CHO cell line.

ChanTest™ hTRPV1-HEK293 cells (Cat # CT6105) are a stably transfected cell line with a human TRPV1 channel constitutively expressed in the HEK 293 cell line.

Both cell lines have been validated by ChanTest for use in manual patch-clamp recording, the PatchXpress® 7000A and IonWorks Barracuda Automated Patch Clamp Systems, and in conjunction with FLIPR® Calcium Kits on the FLIPR® Tetra System

Calcium assay on the FLIPR Tetra Instrument:

For the assay on the FLIPR® Tetra Instrument, cells were washed with Hank's Balanced Salt Solution, treated with trypsin and re-suspended in media without selection antibiotics. Cells were plated at a density of 30,000 cells / well into poly-D-lysine coated 384-well plates and incubated for 18-30 hours in a humidified 5% CO_2, 37ºC cell culture incubator.

Dye loading: For TRPV1 Cells, culture medium was removed and replaced with 20 µL / well FLIPR Calcium 6 dye (Molecular Devices) in HB-PS Buffer. Plates were incubated at 37ºC for 120 minutes. ASIC1a cells were incubated in dye + DPBS at 37ºC for 120 minutes.

Pre-incubation: For TRPV1 cells, a 5X volume of CRC or EC_{80} ligand was prepared in HB-PS 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. For ASIC1a Cells, buffer at 1.5X Log pH was added to cells + dye. ASIC blockers were incubated for 15 min prior of addition of pH 5 buffer.

Data reduction: 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.

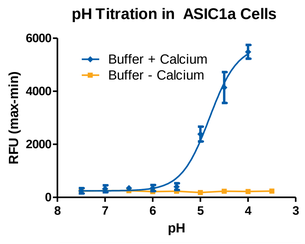
Electrophysiology assay on the IonWorks Barracuda system:

The IonWorks Barracuda System is a fully automated patch clamp system with 384 parallel recording sites, for continuous recording of either voltage- or ligand- gated ion channels. External buffer was first added into the 384-well patch plate, before cells were re-suspended and dispensed into each well. Two seal tests were performed through the application of a 10 mV step, followed by the addition of amphotericin B (100 µg per 1 mL internal solution) as perforating agent to gain access into the cells. Once whole-cell access was achieved, a third seal test was performed before compound(s) were added to the wells, and the recording of the current started. The holding/recording potential was applied continuously at -70 mV for both channel types. All recordings were performed at room temperature.

Results

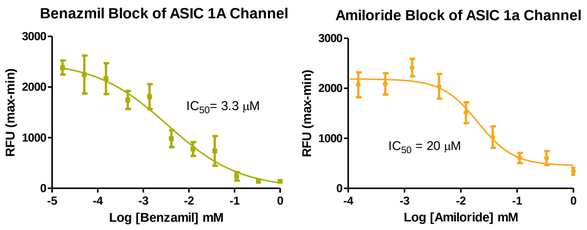
Ligand gated ion channels with calcium sensitive dye

ASIC1a Channel



	Buffer + Ca2+	Buffer - Ca2+
EC_{50} (pH)	5	ND
Z'	0.6	<0.001
n	8	8
Max RFU	5018	236
Min RFU	171	184

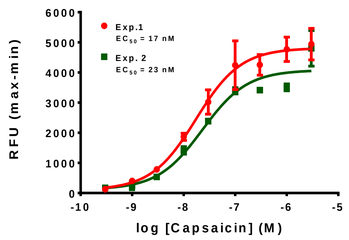
Figure 1. Fluorometric measurement of intracellular calcium triggered by activation of ASIC 1a channel at different pH levels.



	Benzamil	Amiloride
IC_{50} µM	3.3	20
Z'	0.66	0.6
Max RLU	2384	2355
Min RLU	139	344
n=	4	4

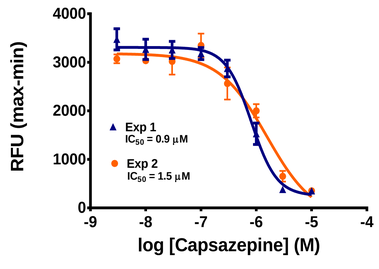
Figure 2. Fluorometric measurement of intracellular calcium triggered by activation of ASIC 1a at pH = 5.0; in the presence of incremental concentrations of ASIC channel blockers.

TRPV1 Channel



Experiment	Mean MAX Signal (RFU*) 3 µM Capsaicin	SD	MIN Signal (RFU*) Vehicle (HB-PS)	SD	Z'	SW
Day 1	4940.81	525.54	125.44	11.87	0.67	6.1
Day 2	4802.82	592.94	172.04	99.53	0.55	4.3

Figure 3. Concentration-dependent activation of TRPV1 channel by capsaicin. Data are Mean ± SD (*n=4*).



Experiment	Mean MAX Signal (RFU*) 10 µM Capsazepine	SD	MIN Signal (RFU*) Vehicle (HB-PS)	SD	Z'	SW
Day 1	3472.56	439.03	351.15	51.25	0.53	3.8
Day 2	3071.82	184.14	352.19	71.94	0.72	10.6

Figure 4. Concentration-dependent inhibition of TRPV1 channel by capsazepine. Data are Mean ± SD (*n=4*).

Electrophysiological characterization of ASIC1a Channel

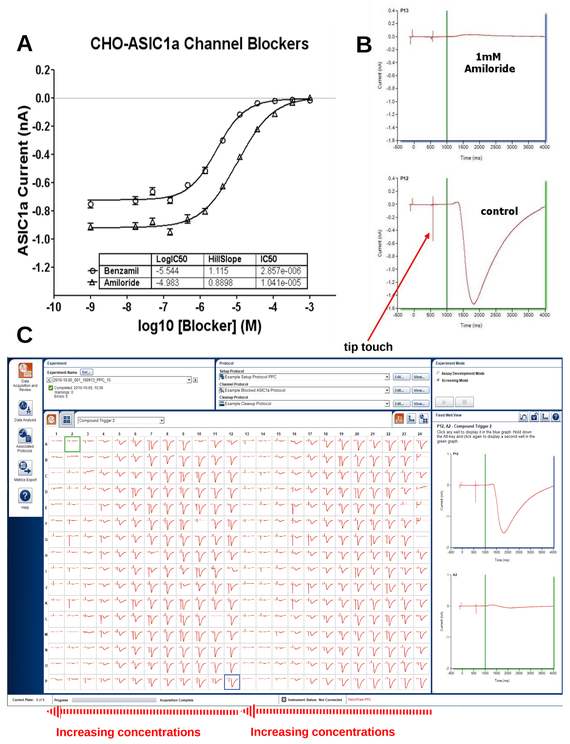


Figure 5. Pharmacological analysis of ASIC1a inhibitors using the IonWorks Barracuda system. A) concentration-dependent inhibition of ASIC1a currents by amiloride and benzamil. Data collected from two different PPC experiments (n = 25-32 for each data points); B) representative ASIC1a currents recorded in the presence and absence of 1mM amiloride; C) plate view of ASIC1a currents obtained on the IonWorks Barracuda system, in response to incremental concentrations of amiloride.

TRPV1 Channel

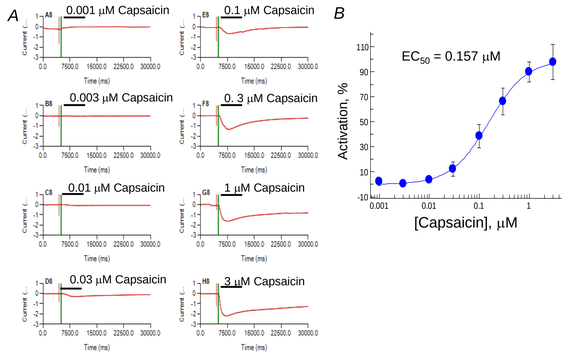


Figure 6. Concentration-dependent activation of TRPV1 Channels by Capsaicin. *A*, representative TRPV1 current traces elicited with 2-second application of capsaicin (0.001 – 3 µM). *B*, a representative dose-response curve of TRPV1 activation with capsaicin. Data presented as Mean ± SD, N=4.

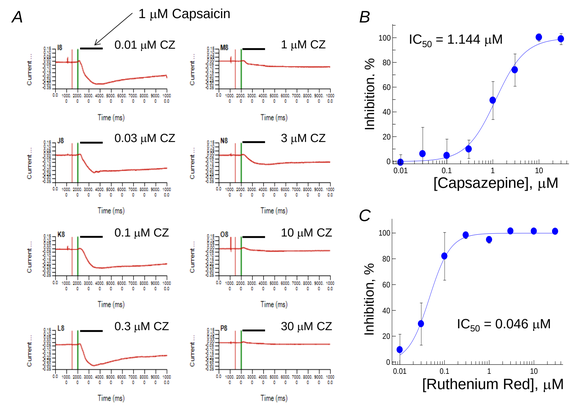


Figure 7. Concentration-dependent Inhibition of TRPV1 channel by capsazepine and Ruthenium Red. *A*, representative TRPV1 current traces elicited with 2-second application of 1 mM capsaicin in the presence of capsazepine (0.01 – 30 mM). Capsazepine was added simultaneously with capsaicin. *B*, dose-response curve of TRPV1 inhibition with capsazepine; data presented as Mean ± SD, N=4. *C*, dose-response curve of TRPV1 inhibition with ruthenium red (ruthenium red was added simultaneously with capsaicin); data presented as Mean ± SD, N=4.

FLIPR Calcium 6 Dye selectivity and toxicity

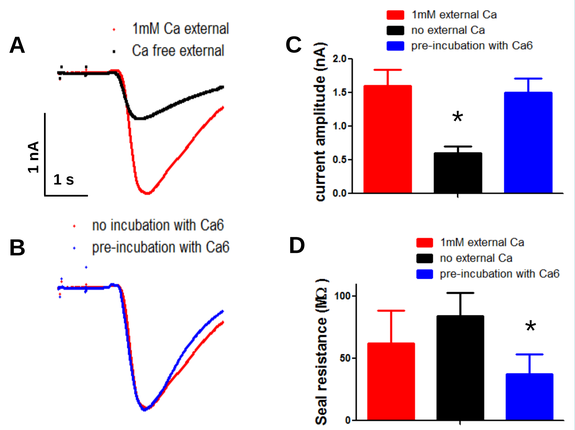


Figure 8. Characterization of calcium selectivity and toxicity of the Ca6 dye. In the first set of experiments the ASIC1a channel currents were recorded in the presence and absence of extracellular calcium. The absolute current amplitude was reduced by ~60% with calcium removed from the external buffer (A, C), whereas the membrane resistance (D), the gating of the channel and the profile of the currents appears unaffected, suggesting that the ASIC1a channels functions normally in the absence of external calcium. In the second set of experiment the cells were pre-incubated with Ca6 dye for 2 hours before being subjected to electrophysiological recording on the IonWorks Barracuda system. The amplitude of channel current was unaffected (B, C) despite a drop of membrane resistance (D), suggesting the Ca6 dye doesn't affect the channel properties within the tested time frame.

Discussion and conclusions

1. **We have validated the utility of a novel calcium sensing dye for sensitive, high-throughput, fluorometric assays of two ligand-gated ion channels.**
2. **The data presented here indicates this novel dye is selective for calcium ions. Under the experimental conditions described in this study, the dye itself does not alter the biophysical properties of the channels.**
3. **The fluorometric assays measure the change of global calcium intracellularly, in response to the activation of ion channels; whereas the electrophysiological assays measure primary signal of ionic movement through the channels. The mechanistic differences between these two methodology could explain the difference of pharmacology presented in this study.**

References

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