Development of Simple, Robust and Highly Sensitive Cell-Based Assays for the Valiscreen™ TRPC3 and TRPC6 Cell Lines

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1 Introduction

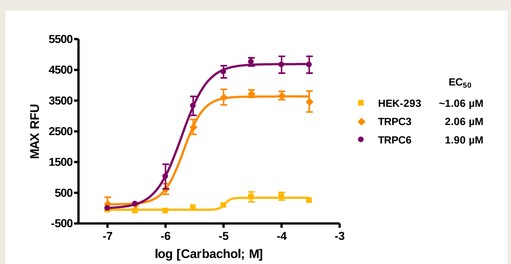
The transient receptor potential (TRP) superfamily consists of monovalent cation and calcium permeable channels. Mammalian TRPs are organized into six families: classical (TRPC), vanilloid (TRPV), melastatin (TRPM), mucolipins (TRPML), polycistin (TRPP) and ankyrin (TRPA). TRPs are expressed in almost every tissue and are involved in a wide range of processes ranging from osmoregulation, thermoregulation, chemical and sensory signaling.

Among the "classical" TRPs, the TRPC family has been proposed to encode components of native store-operated channels and to contribute to transcription factor activation, apoptosis, vascular contractility, platelet activation, cardiac hypertrophy, as well as cell proliferation. In particular TRPC3 and TRPC6 emerge as important targets of pharmacological intervention that may ameliorate complex human diseases. TRPC6 is a target of hyperforin, which is the active ingredient of the antidepressive St. John's wort (*Hypericum perforatum*).

ValiScreen™ human TRPC3 and TRPC6 recombinant HEK-293 cell lines (PerkinElmer) were developed and characterized in cell-based assays using Membrane Potential fluorescent dyes on a FLIPR$^{TETRA®}$ system (Molecular Devices), and manual patch-clamp. In these assays, the TRPC3 and 6 channels were activated via G-protein-coupled receptor activation by Carbachol and the subsequent phospholipase C activation and intracellular calcium stores depletion.

2 Carbachol Response in Transfected and Parental Cell Lines

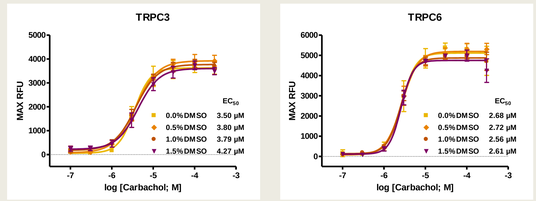
Carbachol activator dose-response was analyzed in wild-type and in transfected HEK-293 TRPC3 and TRPC6 cells. Cells were plated at a density of 20,000 cells per well and analyzed 24 hr later in the Membrane Potential Assay.
The dose-dependent Carbachol response observed in TRPC3 and TRPC6 cells was TRPC specific, as no significant Carbachol response was observed in the HEK-293 parental cell line.



Membrane Potential Assay on WT and TRPC stable cells lines

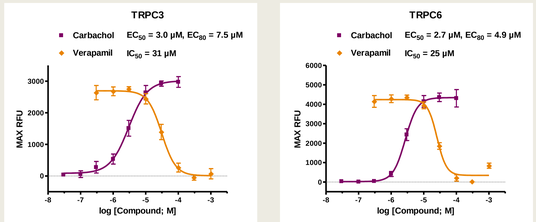
3 Tolerance to DMSO

DMSO was first injected on cells, and after a 5-min incubation, the Carbachol response was analyzed in the Membrane Potential Assay. DMSO had no effect on the assay signal or pharmacology at all concentrations tested.



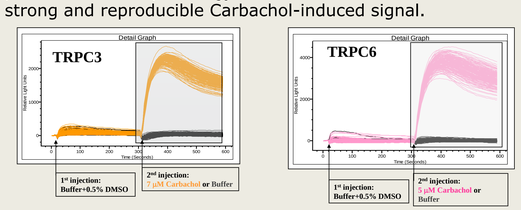
4 Pharmacology

Carbachol activator EC_{50} and Verapamil channel blocker IC_{50} values were determined in the Membrane Potential Assay for both the TRPC3 and TRPC6 cell lines. For the channel blocker assays, a concentration of 10 µM Carbachol was used to stimulate the cells.



5 Reproducibility

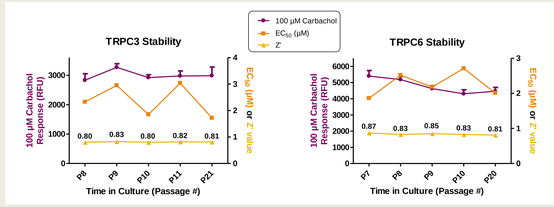
Reproducibility of the TRPC3 and TRPC6 Membrane Potential Assays was examined by testing 2 full 384-well plates per day on 3 different days, using Carbachol's EC_{80} vs buffer (n = 2 x160 points) for Z' calculation, and a Carbachol Dose Response (n = 8 per concentration) for EC_{50} calculation. Both cell lines exhibited a strong and reproducible Carbachol-induced signal.



		TRPC3		TRPC6	
		EC_{50} (µM)	Z' (buffer vs EC_{80})	EC_{50} (µM)	Z' (buffer vs EC_{80})
Day 1	plate 1	2.33	0.80	1.86	0.87
	plate2	3.00	0.77	1.96	0.84
Day2	plate 1	2.95	0.83	2.53	0.83
	plate2	3.69	0.85	2.22	0.83
Day 3	plate 1	1.85	0.80	2.18	0.85
	plate2	2.43	0.90	2.41	0.86

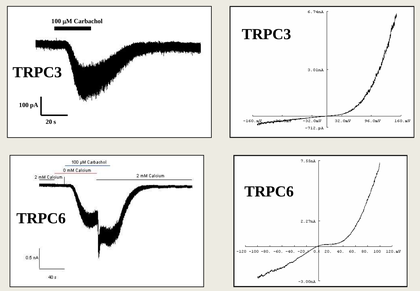
6 Stability over Time in Culture

Stability of the absolute response signal, sensitivity to Carbachol and assay quality were evaluated in TRPC3 and TRPC6 cells cultivated up to passage 20. Both cell lines exhibited strong and reproducible Carbachol-induced signal for up to 20 passages.



7 TRPC3 and TRPC6 Electrophysiology

Application of 100 µM Carbachol resulted in a slow activation/deactivation kinetics and in a progressive desensitization (not shown). For TRPC6, the presence of extracellular calcium enhanced the extent of activation and accelerated the deactivation process.
For both channels, ramps from -150 mV to +150 mV were applied for 300 ms after the application of 100 µM Carbachol: the elicited outward rectifying currents reversed close to 0 mV suggesting the opening of a non-selective cation channel.



8 Methods

Membrane Potential Dyes Measurement
15,000 cells/well (unless otherwise indicated) were seeded in black-walled clear-bottom Poly-D-Lysine coated 384-MTPs. 24 hr after seeding, cells were loaded with Membrane Potential Sensitive Blue Dye (Molecular Devices) solubilized in Low Calcium Tyrode's buffer, in the presence of 30 µM BAPTA-AM (Invitrogen Corporation). Fluorescence was monitored using the FLIPR$^{TETRA®}$ instrument (Molecular Devices) following a double injection kinetic: 1st injection: compound solutions, in Low Calcium Tyrode's buffer and 0.5% DMSO; 2nd injection: Carbachol solutions, in Low Calcium Tyrode's buffer. Measurements were analyzed with Screenworks© software (Molecular Devices, Version 2.0.0.24) and data were exported as Maximum (MAX) Statistics calculated from sample 64 (Start Reading Time of 2nd injection) to sample 123 (End Read=300 s after 2nd injection), after applying "Subtract Bias on Sample: 1" and "Spatial Uniformity" corrections. Mean, standard deviation and Z' values were calculated on the exported data with Excel software. Values were then used to create sigmoidal dose-response curves (variable slope) with GraphPad PRISM® software (Version 4.03).

Clone selection
TRPC3- or TRPC6-transfected HEK-293 cells were selected with G418 and underwent two rounds of limiting dilutions. The clones having the best Carbachol response in the FLIPR® Membrane Potential Sensitive Dye assay were selected (AX-011-C and AX-012-C ValiScreen™ cell lines, PerkinElmer).

Electrophysiological recording and Analysis
Experiments were performed through patch-clamp technique in whole-cell voltage clamp configuration.
Recording solutions were as follows:
- Extracellular (1) 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.4 with NaOH;
- Extracellular (2) 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, pH 7.4 with NaOH
- Intracellular 128 mM CsCl, 12 mM EGTA, 3 mM $MgCl_2$, 0.7 mM $CaCl_2$, 10 mM HEPES, 5 mM Na_2ATP, pH 7.2 with CsOH.

For data acquisition and further analysis, the EPC10 digitally controlled amplifier, in combination with PATCHMASTER software (HEKA Electronics, Lambrecht, Germany), was used. Currents were elicited by application of extracellular Carbachol in continuous recording mode (-80 mV). Voltage ramps (Vm = 0 mV) from -150 mV to +150 mV over 300 ms were applied before and after the application of Carbachol.

9 References

1. Watanabe H *et al. Pharmacol. Ther.* (2008) **118**:337-51
2. Eder P *et al. Exp Pharmacol.* (2007) **179**:77-92
3. Eder P, Groschner K *Channels (Austin)* (2008) **2**(2)
4. Lemonnier L *et al. Cell Calcium.* (2008) **43**:506-14

10 Concluding Remarks

1. ValiScreen stable HEK-293 cell lines overexpressing human TRPC3 and TRPC6 were generated.
2. A Membrane-Potential Fluorescent Dye assay was developed to characterize their pharmacological properties with both the channel activator Carbachol and blocker Verapamil. This assay was run on an automated robotic screening platform (FLIPR$^{TETRA®}$).
3. Carbachol EC_{50} and Verapamil IC_{50} value reproducibility, tolerance to DMSO, assay robustness and the stability of the response of the two cell lines over time were determined.
4. The TRPC3 and TRPC6 electrophysiological properties were assessed by patch-clamp experiments.
5. The ValiScreen TRPC3 and TRPC6 assays were run in a miniaturized format providing simple, robust, highly sensitive functional assays suitable for studying cations influx mediated by TRPC activation and identification of TRPC3 and TRPC6 modulators in HTS campaigns.

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