B'SYS

B'SYS GmbH

HEK-293 TRPA1 Cell Line

1 MATERIAL AND METHODS 3
2 AGONISTS 4
3 ANTAGONISTS 5
4 CELL CULTURE CONDITIONS 6
4.1 GENERAL 6
4.2 RECOMMENDED COMPLETE MEDIUM 6
4.3 ANTIBIOTICS 6
4.4 THAWING CELLS 6
4.5 SPLITTING CELLS 6
4.6 FREEZING MEDIUM 7
4.7 FREEZING CELLS 7
5 TRPA1 SEQUENCE 7
5.1 HUMAN TRPA1 ACCESSION NUMBER NP_015628 7
6 CONTACT INFORMATION 8
6.1 CONTACT ADDRESS FOR TECHNICAL SUPPORT AND ORDERING INFORMATION 8

1 MATERIAL AND METHODS

For manual patch-clamping a setup equipped with an EPC-10 amplifier and Patchmaster Software was used. For automated patch-clamping a 16 channel Q-Patch system was used.

Cells were clamped at -80 mV and voltage ramps (-100 to +60 mV, 2 s) were applied every 10 s. Current amplitudes at -100 mV and +60 mV were analyzed, averaged and normalized to control conditions.

The extracellular solution contained (in mM) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 10, Hepes 10, D-Glucose 10. The pH was adjusted to 7.4 with NaOH. The intracellular solution consisted of (in mM) KCl 130, MgCl2 1, Mg-ATP 5, EGTA 5. The pH was adjusted to 7.3 with KOH. After formation of a GΩ seal between the patch electrodes and individual TRPA1 stably transfected HEK TRPA1 cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. All solutions applied to cells were continuously perfused and maintained at room temperature. As soon as a stable seal could be established the cells were clamped at -80 mV and the voltage stimulus was applied continuously.

2 AGONISTS

TRPA1 channels were activated with increasing concentrations of Supercinnamaldehyde (0.03 µM – 1.0 µM) or AITC (0.01 µM – 10 µM). Due to desensitisation at high concentrations the EC_{50} of Supercinnamaldehyde could not be determined, for AITC the IC_{50} was calculated to be 2.12 µM.

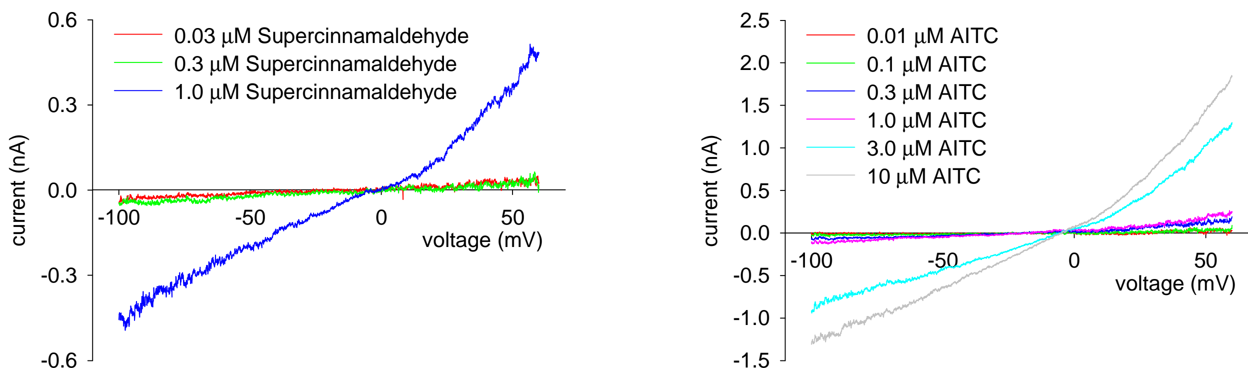

Fig.1: Representative current recordings for HEK TRPA1 cells treated with increasing concentrations of Supercinnamaldehyd or AITC. Currents of voltage ramps at different concentrations were superimposed. Endogenous background current (recorded under control conditions) were subtracted.

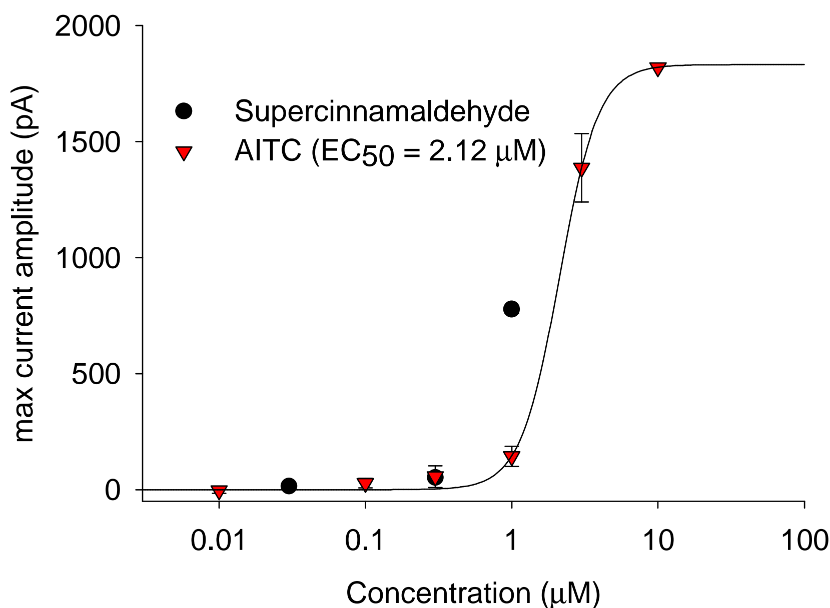

Fig.2: Dose response curves for Supercinnamaldehyde and AITC. Due to desensitisation of currents at concentrations higher than 1 µM of Supercinnamaldehyd, no EC_{50} could be determined. (current amplitudes at the maximal current amplitude were averaged for at least n=2 cells and plot versus the perfused concentration of agonist. Data were fit with a 3 parameter logistic equation)

3 ANTAGONISTS

The unselective TRP antagonist Ruthenium Red was used to test the block of TRPA1 channels stimulated with 1 µM Supercinnamaldehyde. The maximal current amplitude of each voltage ramp was normalized to the maximal current amplitude under control conditions. The IC_{50} of Ruthenium Red was determined to be 12.25 nM.

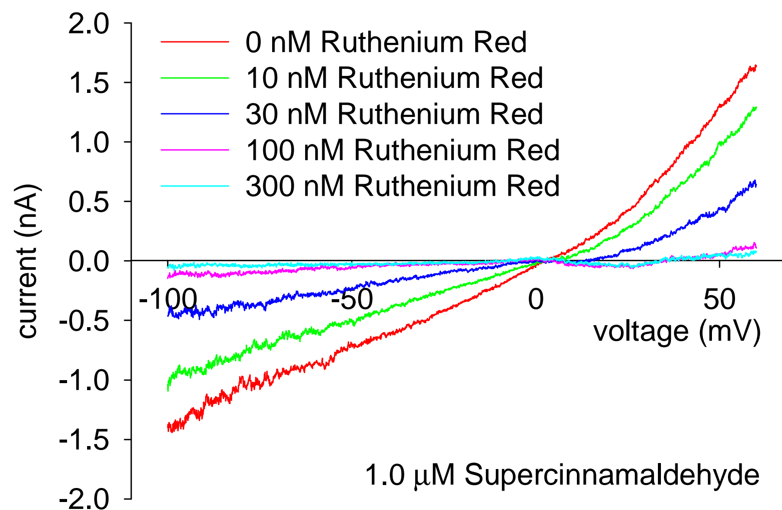

Fig.3: Representative current recordings for HEK TRPA1 cells treated with increasing concentrations of Ruthenium Red in the presence of 1 µM Supercinnamaldehyd. Currents of voltage ramps at different concentrations were superimposed. Endogenous background current (recorded under control conditions) were subtracted.

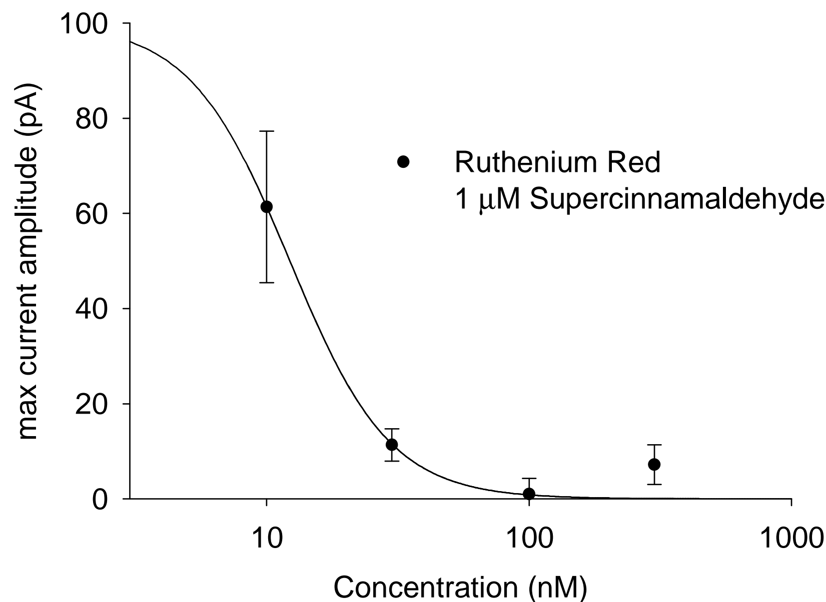

Fig.4: Dose response curve for Ruthenium Red at the presence of 1 µM Supercinnamaldehyde (current amplitudes at the maximal current amplitude were averaged for at least n=2 cells and plot versus the perfused concentration of Ruthenium Red. Data were fit with a 3 parameter logistic equation)

4 CELL CULTURE CONDITIONS

4.1 General

HEK TRPA1 cells are incubated at 37°C in a humidified atmosphere with 5% CO_2 (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing DMEM medium supplemented with 10% fetal bovine serum, 1.0% Penicillin/Streptomycin solution and 100 µg/mL Hygromycin / 15 µg/mL Blasticidin. The HEK TRPA1 cells are passaged at a confluence of about 80%. For electrophysiological measurements the cells are seeded onto e.g. 35 mm sterile culture dishes containing complete medium.

- All solutions and equipment coming in contact with the cells must be sterile.
- Use proper sterile technique and work in a laminar flow hood.
- Be sure to have frozen cell stocks at hand before starting experiments.
- Cells should be split every 2-3 days at 70% - 80% confluency at 1:3 to 1:5 ratio.

EXPRESSION OF TRPA1 HAS TO BE INDUCED BY ADDING 2.5 µg/mL TETRACYCLINE AT LEAST 24 H BEFORE START OF EXPERIMENTATION

4.2 Recommended Complete Medium

- DMEM with Glutamine or GlutaMAX I
- 10% FBS
- 1.0% Penicillin/Streptomycin

4.3 Antibiotics

- HEK TRPA1 clones were selected under 500 µg/mL Hygromycin / 15 µg/mL Blasticidin antibiotic pressure.
- To cultivate HEK TRPA1 cells, 100 µg/mL Hygromycin / 15 µg/ml Blasticidinare recommenden.
- To separate HEK TRPA1 cells from untransfected cells, use 500 µg/mL Hygromycin / 15 µg/mL Blasticidin

Remark: The permanent application of high antibiotic pressure has no effect on current density.

4.4 Thawing Cells

- Remove vial of cells from liquid nitrogen and thaw quickly at 37°C.
- Decontaminate outside of vial with 70% ethanol.
- Transfer cells to a T-75 culture flask containing 10 mL complete medium.
- Incubate cells at 37°C for 4-6 h to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 10 mL complete medium & antibiotics.
- Antibiotics: 100 µg/mL Hygromycin / 15 µg/mL Blasticidin.
- Incubate cells and check them daily until 70% - 80% confluency is reached.

4.5 Splitting Cells

- When cells are 70% - 80% confluent remove medium.
- Wash cells once with 1x PBS to remove excess medium.

- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Passage cells into new flask with complete medium and antibiotics at 1:3 to 1:5 ratio.

4.6 Freezing Medium

- Mix 0.9 mL fresh complete medium and 0.1 mL DMSO for every 1 mL freezing medium.
- Sterilize freezing medium by means of appropriate micro filter (0.1 µm – 0.2 µm).

4.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluency prior to freezing.
- Remove the complete medium.
- Wash cells once with 1x PBS to remove excess medium.
- Add 1x Trypsin/EDTA and incubate 30 s at room temperature.
- Remove Trypsin/EDTA quickly and incubate cells for 2 min at 37°C.
- Detach cells, add complete medium and pipet up and down to break clumps of cells.
- Pellet cells with centrifuge and carefully aspirate off medium.
- Resuspend cells at a density of approximately 2.0 E+06 cells per mL with fresh freezing medium.
- Aliquot 0.5 mL of cell suspension into each cryovial.
- Overnight incubate cells in a styropor box at –80°C.
- The next morning transfer cryovial in liquid nitrogen tank for long-term storage.

5 TRPA1 SEQUENCE

5.1 Human TRPA1 Accession Number NP_015628

Cloned cDNA sequence of human TRPA1 subunit was error-free and encodes for NP_015628:

```
MKRSLRKMWRPGEKKEPQGVVYEDVPDDTEDFKESLKVVFEGSAYGLQNFNKQKKLKRCDDMDTFFLHYAAAEGQIELME
KITRDSSLEVLHEMDDYGNTPLHCAVEKNQIESVKFLLSRGANPNLRNFNMMAPLHIAVQGMNNEVMKVLLEHRTIDVNL
EGENGNTAVIIACTTNNSEALQILLKKGAKPCKSNKWGCFPIHQAAFSGSKECMEIILRFGEEHGYSRQLHINFMNNGKA
TPLHLAVQNGDLEMIKMCLDNGAQIDPVEKGRCTAIHFAATQGATEIVKLMISSYSGSVDIVNTTDGCHETMLHRASLFD
HHELADYLISVGADINKIDSEGRSPLILATASASWNIVNLLLSKGAQVDIKDNFGRNFLHLTVQQPYGLKNLRPEFMQMQ
QIKELVMDEDNDGCTPLHYACRQGGPGSVNNLLGFNVSIHSKSKDKKSPLHFAASYGRINTCQRLLQDISDTRLLNEGDL
HGMTPLHLAAKNGHDKVVQLLLKKGALFLSDHNGWTALHHASMGGYTQTMKVILDTNLKCTDRLDEDGNTALHFAAREGH
AKAVALLLSHNADIVLNKQQASFLHLALHNKRKEVVLTIIRSKRWDECLKIFSHNSPGNKCPITEMIEYLPECMKVLLDF
CMLHSTEDKSCRDYYIEYNFKYLQCPLEFTKKTPTQDVIYEPLTALNAMVQNNRIELLNHPVCKEYLLMKWLAYGFRAHM
MNLGSYCLGLIPMTILVVNIKPGMAFNSTGIINETSDHSEILDTTNSYLIKTCMILVFLSSIFGYCKEAGQIFQQKRNYF
MDISNVLEWIIYTTGIIFVLPLFVEIPAHLQWQCGAIAVYFYWMNFLLYLQRFENCGIFIVMLEVILKTLLRSTVVFIFL
LLAFGLSFYILLNLQDPFSSPLLSIIQTFSMMLGDINYRESFLEPYLRNELAHPVLSFAQLVSFTIFVPIVLMNLLIGLA
VGDIAEVQKHASLKRIAMQVELHTSLEKKLPLWFLRKVDQKSTIVYPNKPRSGGMLFHIFCFLFCTGEIRQEIPNADKSL
EMEILKQKYRLKDLTFLLEKQHELIKLIIQKMEIISETEDDDSHCSFQDRFKKEQMEQRNSRWNTVLRAVKAKTHHLEP
```

6 CONTACT INFORMATION

6.1 Contact Address for Technical Support and Ordering Information

- B'SYS GmbH
  Technology Center Witterswil
  Benkenstrasse 254
  4108 Witterswil
  Switzerland

  Tel: +41 61 721 77 44
  Fax: +41 61 721 77 41
  Email: info@bsys.ch
  Web: www.bsys.ch