Expression and purification of the human TRPV1 for structural studies.

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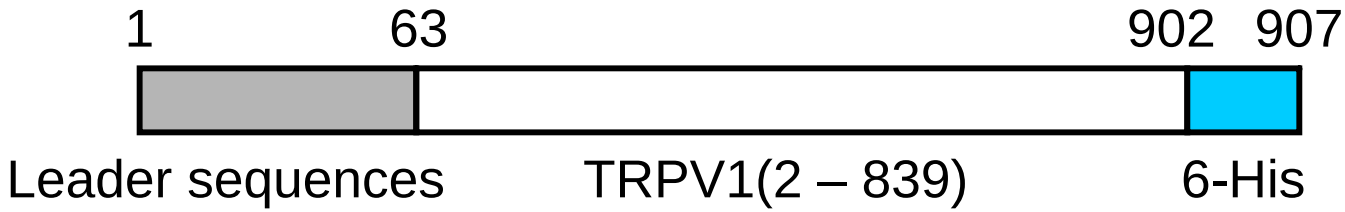
TRPV1 is a transmembrane cation channel with high permeability to Ca^{2+} that is activated by multiple stimuli such as the vanilloid compound capsaicin, noxious heat, and low pH values. This channel is involved in acute thermal nociception and neurogenic inflammation.

As a first step towards structural analyses of human TRPV1, full-length human TRPV1 with a hexahistidine tag was over-expressed using a baculovirus-infected High Five cells. Functional activity of membrane-expressed TRPV1 was confirmed by specific ligand binding and whole-cell ligand-gated current recordings.

Efficient solubilization and purification protocols were developed to yield milligram quantities of detergent-solubilized TRPV1 at 80-90% homogeneity. Western blots, mass spectrometric analyses and specific ligand binding of purified TRPV1 confirmed the preservation of protein integrity.

TRPV1 Heterologous Expression

Schematic representation of TRPV1 expression construct.

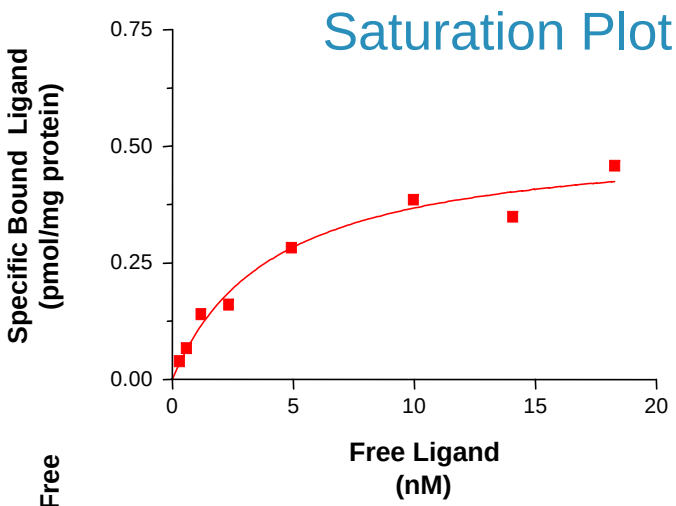


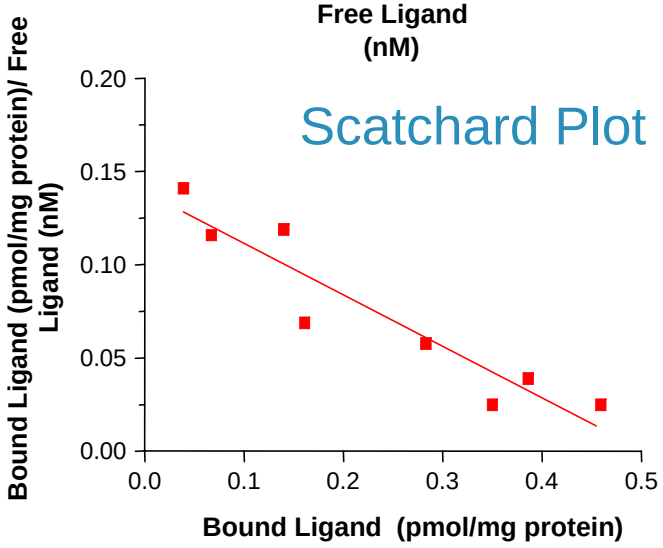
Total number of amino acid residues in the expressed protein is 907 with 838 amino acids corresponding to full-length human TRPV1 flanked by hexahistidine tag and leader sequences.

Protein expression was driven by the polyhedrin promoter.

Protein expressed in High Five insect cells (*Trichoplusia ni)* using Wave Bioreactors (Wave Biotech) and Express Five SFM Media. Cells (2.2 x 10^6 cells/ml) were infected with recombinant virus at multiplicity of infection of 1 and harvested 48 hours later.

TRPV1 Ligand Binding of Isolated Membranes





High Five cell membranes in 50 mM Tris, pH7.4 were incubated with [^{3}H] A-778317 for 60 min at 22^{o}C.

Non-specific binding was defined by the addition of 1 μM of unlabeled ligand.

Free and bound ligands were separated by filtration through Whatman Glass Fiber Filter (GF/B). Radioactivity was counted in Beckman LS6500 scintillation counter.

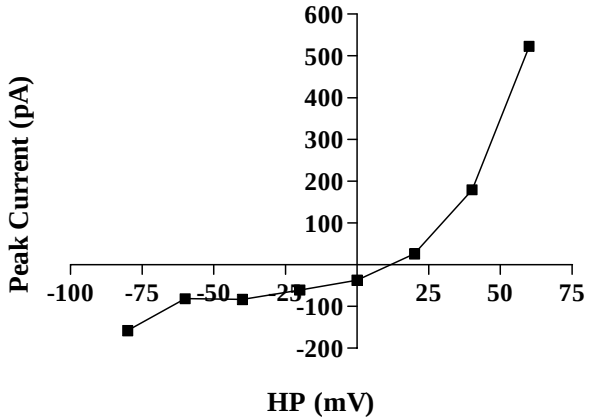
Tight binding:

K_D = 4.04 $\pm$ 1.08 nM

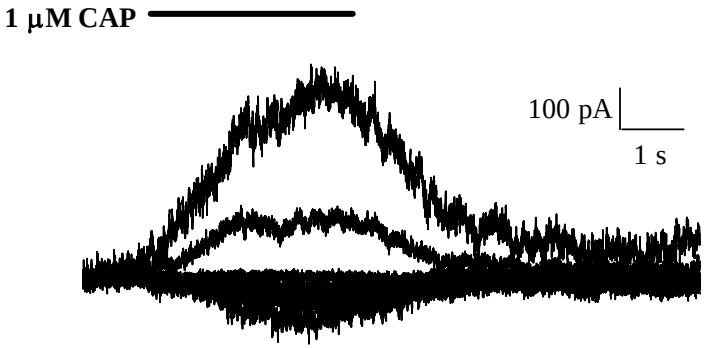
B_{max} = 0.502 $\pm$ 0.11 pmol/mg of protein

TRPV1 Channel Activity in High Five Cells

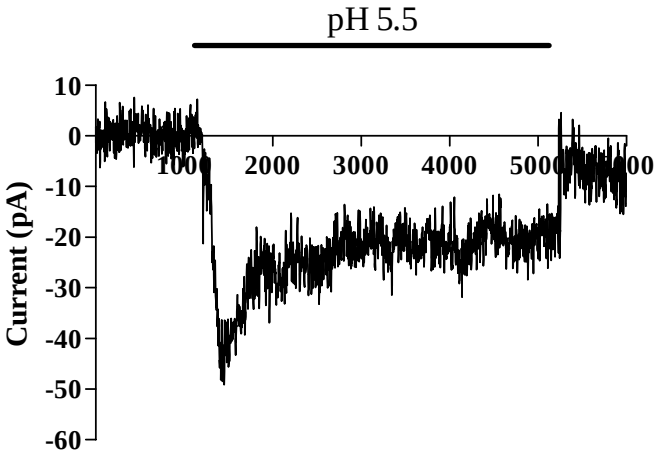
Current / Voltage Relations of Capsaicin-Activated TRPV1



Current response to capsaicin



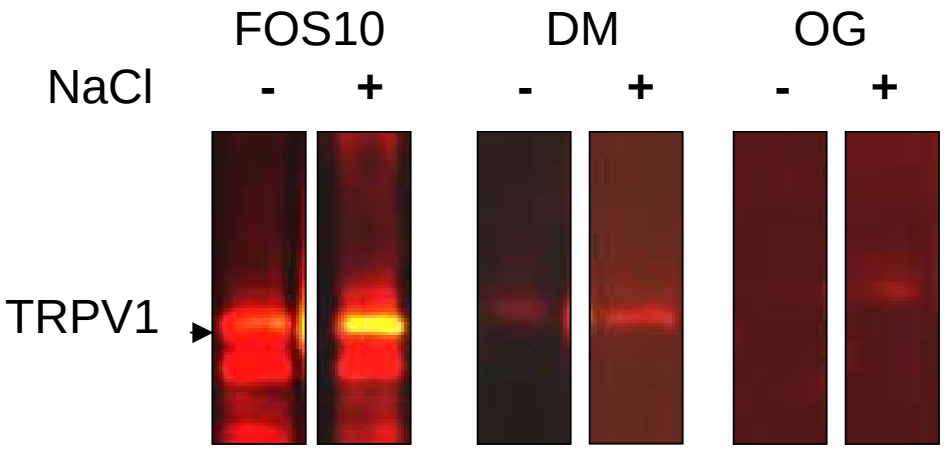
Current response to acidic pH



- The larger current at positive potentials (+ 60 mV) is the outward rectification typical of TRPV1
- 100 pA inward current induced by 1 µM capsaicin
- Acidic pH generates rapidly deactivating inward current typical of TRPV1 acid activated currents

TRPV1 Solubilization Screening

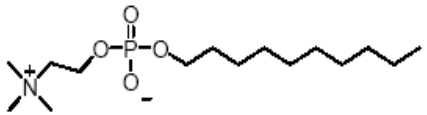
Sample of small scale solubilization screening of TRPV1



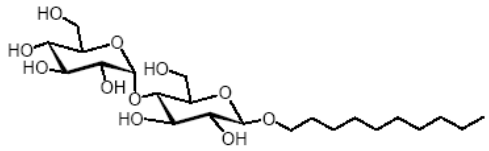
Western blots of samples detected by TRPV1 ABRK-1 and C-terminal His-tag antibody. Developed with secondary antibodies conjugated with two type of fluorophores. Yellow color corresponds to the overlapping fluorophore detection

Tested: 13 detergents +/- 0.5 M NaCl

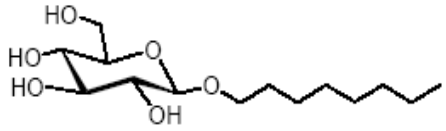
Shown are solubilization results for:
Foscholine 10 (FOS10),
DecylMaltoside (DM),
and Octyl Glucoside (OG)



FOS10



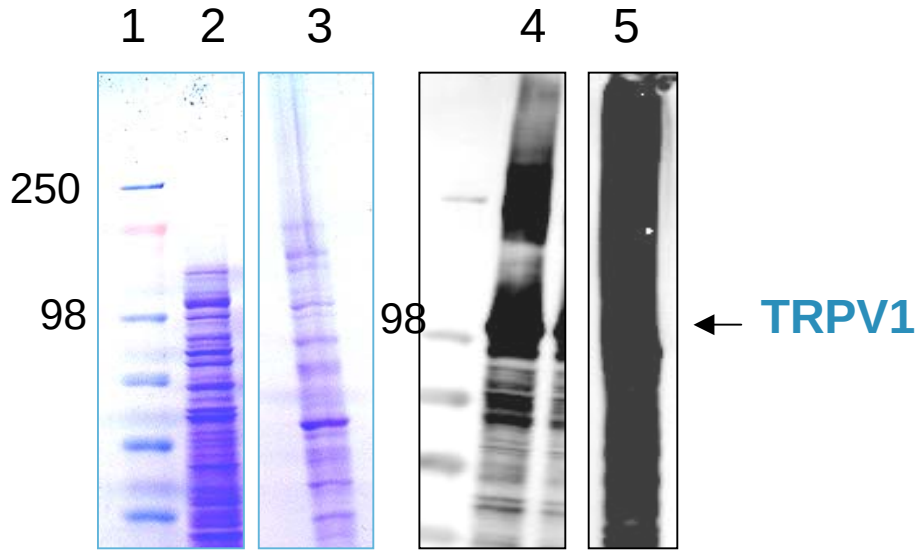
DM



OG

Best solubilization yield with Foscholine 10 and Foscholine 12 in presence of high salt and urea

TRPV1 Large Scale Solubilization



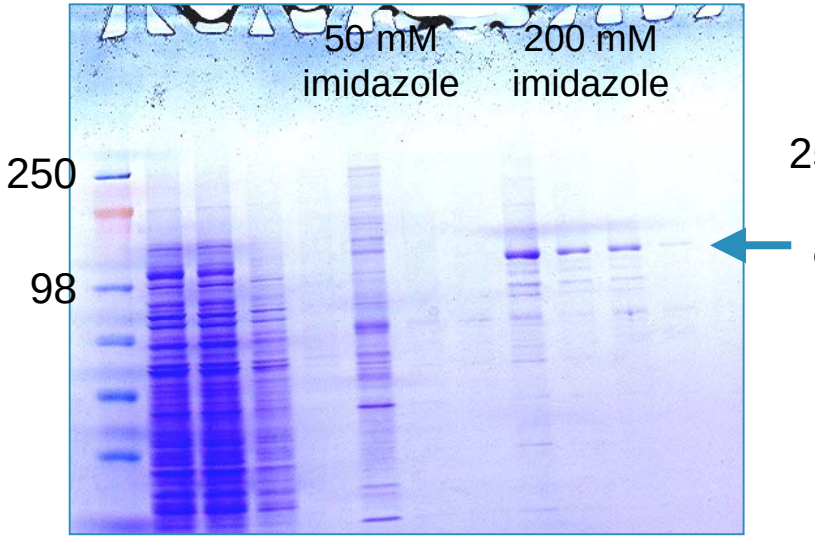
Lane 1	Molecular weight markers, kDa
Lanes 2 and 3	Coomassie R-250 stained SDS/PAGE of supernatant (2) and pellet (3) after ultracentrifugation to separate solubilized and non-solubilized material
Lanes 4 and 5	Western blots of the samples shown on lanes 2 and 3 probed with TRPV1 ABRK-1 Ab

TRPV1 Purification: Ni Affinity Column

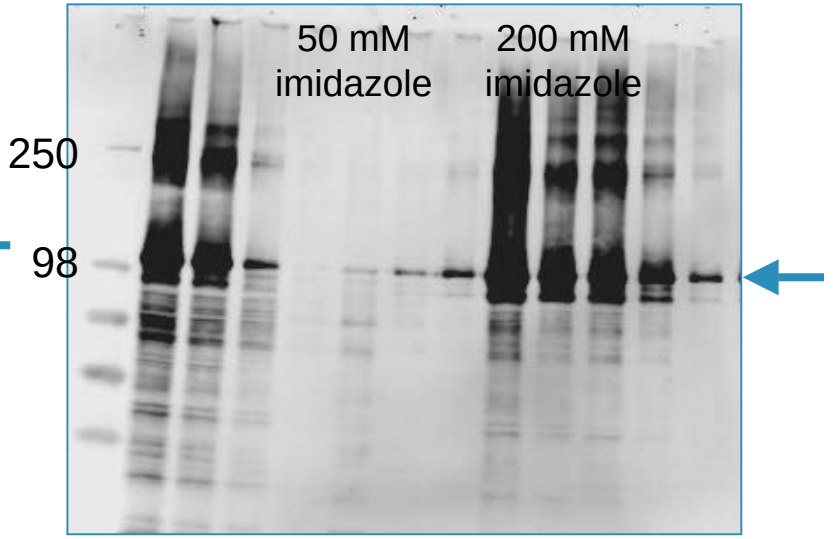
- Column ran in 50 mM Tris, pH 7.4, 0.5 M Urea, 25 mM Glycine, 0.5 M NaCl, 0.5 mM TCEP, 0.05% Foscholine 12, 5 mM Imidazole.

- Column washed with 50 mM Imidazole.

- Protein eluted with 200 mM Imidazole.



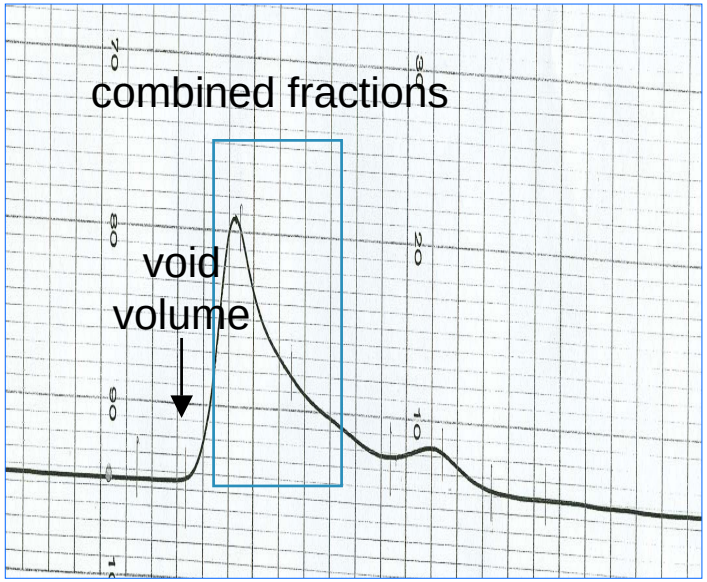
4 – 20% Tris-Glycine SDS PAGE



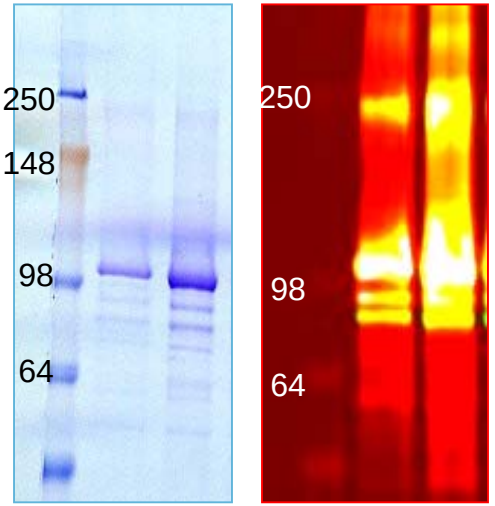
Western Blot of the gel: ABRK1 AB

TRPV1 Purification: Superdex S200 Column

Column buffer: 50 mM Tris, pH 7.4, 0.5 M NaCl, 0.5 mM TCEP, 0.05% Foscholine 12, 5 mM Spermine.



HiLoad 26/60 Superdex 200 chromatogram.



Combined and concentrated fractions. Western detection with ABRK1 and anti His-tag Abs

TRPV1 Characterization: Mass Spec Analysis

Leader sequences: GP67 signal peptide TEV Thrombin

MLLVNQSHQGFNMVSAIVLYVLLAAAAHSAFA*ENLYFQ*GKLITSLYKKAGF*LVPRGS*

2 KKWSSTDLG AAADPLQKDT CPDPLDGDPN SRPPPAKPQL STAKSRTRLF GKGDSEEAFP

61 VDCPHEEGEL DSCPTITVSP VITIQRPGDG PTGARLLSQD SVAASTEKTL RLYDRRSIFE

121 AVAQNNCQDL ESLLLFLQKS KKHLTDNEFK DPETGKTCLL KAMLNLHDGQ NTTIPLLLEI

181 ARQTDSLKEL VNASYTDSYY KGQTALHIAI ERRNMALVTL LVENGADVQA AAHGDFFKKT

241 KGRPGFYFGE LPLSLAACTN QLGIVKFLLQ NSWQTADISA RDSVGNTVLH ALVEVADNTA

301 DNTKFVTSMY NEILILGAKL HPTLKLEELT NKKGMTPLAL AAGTGKIGVL AYILQREIQE

361 PECRHLSRKF TEWAYGPVHS SLYDLSCIDT CEKNSVLEVI AYSSSETPNR HDMLLVEPLN

421 RLLQDKWDRF VKRIFYFNFL VY**C**LYMIIFT MAAYYRPVDG LPPFKMEKTG DYFRVTGEIL

481 SVLGGVYFFF RGIQYFLQRR PSMKTLFVDS YSEMLFFLQS LFMLATVVLY FSHLKEYVAS

541 MVFSLALGWT NMLYYTRGFQ QMGIYAVMIE KMILRDLCRF MFVYIVFLFG FSTAVVTLIE

601 DGKNDSLPSE STSHRWRGPA CRPPDSSYNS LYSTCLELFK FTIGMGDLEF TENYDFKAVF

661 IILLLAYVIL TYILLLNMLI ALMGETVNKI AQESKNIWKL QRAITILDTE KSFLKCMRKA

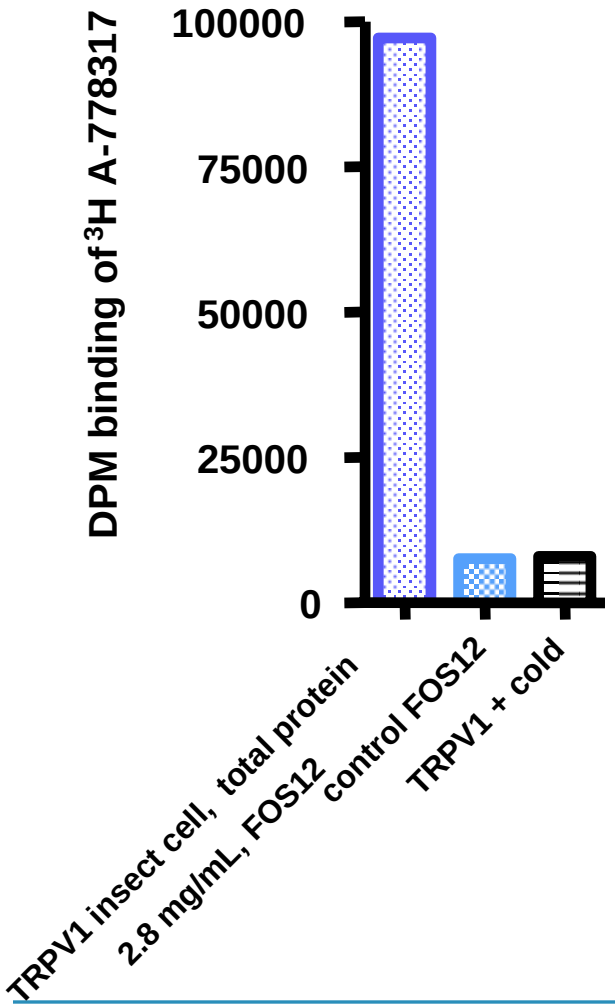
721 FRSGKLLQVG YTPDGKDDYR WCFRVDEVNW TTWNTNVGII NEDPGNCEGV KRTLSFSLRS

781 SRVSGRHWKN FALVPLLREA SARDRQSAQP EEVYLRQFSG SLKPEDAEVF KSPAASGEK

HHHH

- MS analysis of tryptic in-gel digestion of TRPV1 band and database search identified ~ 50% of primary amino acids (blue color).
- Less than 2% are within TM regions (underlined)
- Leader peptide is not cleaved posttranslationally.

TRPV1 Characterization: Ligand Binding of Purified Protein



Purified TRPV1 binds [^{3}H]-A-778317

TRPV1 in 50 mM Tris, pH 7.4, 0.5 M NaCl, 5% Glycerol, 0.5 mM TCEP, 0.05% FOS12 was incubated with [^{3}H] A-778317 for 2 h at 4^{o}C.

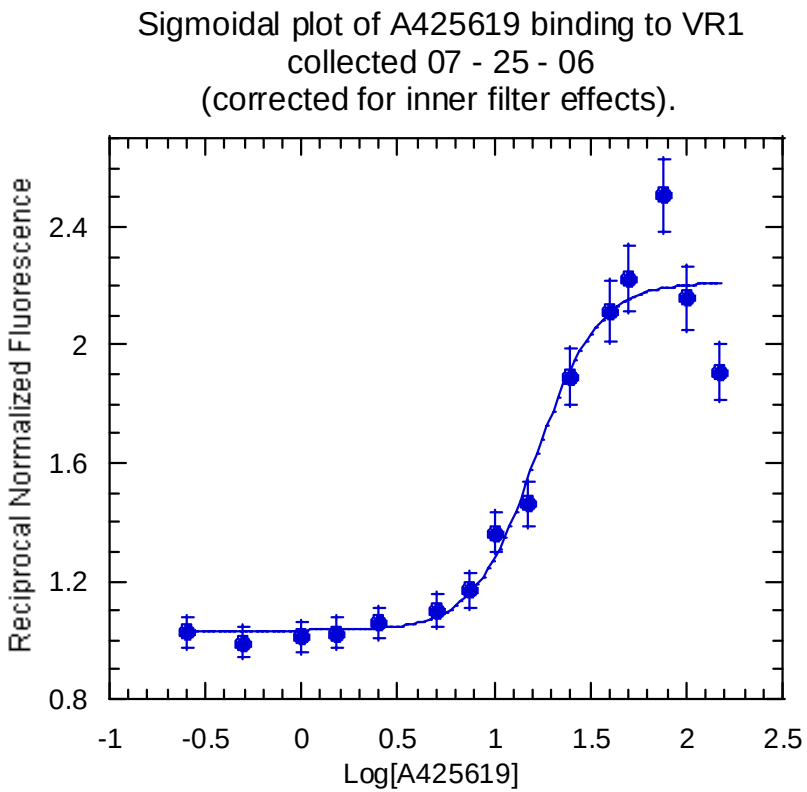
Non-specific binding was defined by the addition of 10 μM of unlabeled A-425619 ligand.

Free and bound ligands were separated by using Econo-Pac 10DG desalting columns.

Radioactivity was counted in Beckman LS6500 scintillation counter.

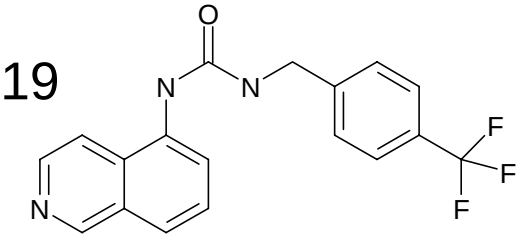
Protein Characterization

Fluorescent Ligand Binding Assay to characterize purified protein. Used FRET (Förster Fluorescence Resonance Energy Transfer).



Kd = 16.6 μM

A-425619



Tryptophan - Donor:
excitation at 280 nm,
emission max at 330 - 350 nm

A-425619 - Acceptor:
excitation at 320 - 340 nm,
emission max at 450 - 500 nm.

Monitor the change in protein fluorescence upon ligand binding

Conclusions

- TRPV1 was heterologously expressed in High Five insect cells

- Functional activity of expressed TRPV1 was confirmed by saturation ligand binding and activation by capsaicin and low pH as shown by whole cell voltage clamp measurements.

- TRPV1 solubilization and purification procedure was developed. It allows to purify 5 mg of active TRPV1 protein out of 20 g of cell paste.