

B'SYS GmbH

HEK-293 Ca_v1.2 Cell Line

Specification Sheet

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1 BACKGROUND

1.1 The cardiac L-type I_{Ca} current is encoded by Cav1.2

Around 20 different ionic currents account for the cardiac action potential. Briefly, these can be divided into depolarizing and repolarizing currents. The plateau phase of the cardiac action potential is mainly maintained by inward calcium flux through voltage-gated L-Type Cav1.2 channels. Mutations in the CACNA1C gene can cause the Long QT Syndrome with Syndactyly; Timothy Syndrome.

1.2 B'SYS' HEK-293 Cav1.2 Assay

B'SYS has designed a HEK-293 Cav1.2 cell line with stable expression of human Cav1.2 calcium channels. The human Cav1.2 α , $\beta 2$ and $\alpha 2\delta 1$ subunits cDNA were cloned and cotransfected into HEK-293 cells and then the functional properties of the Cav1.2 channels validated by means of the patch-clamp technique. Results are outlined in section 2.

B'SYS' HEK-293 Cav1.2 are suitable and were validated using manual and automated patch-clamping (Sophion Qube and Sophion Q-Patch, see data below).

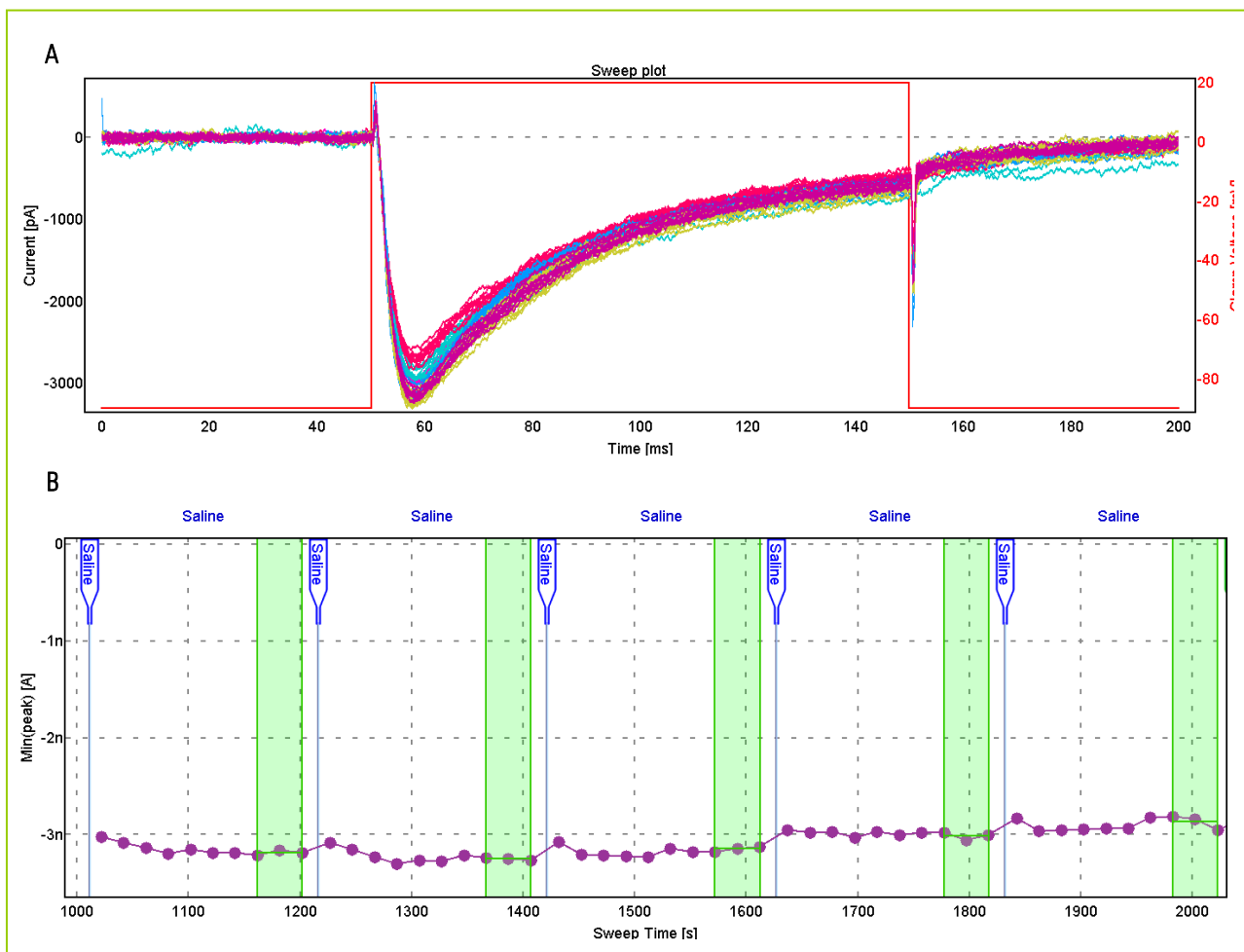


Fig. 1: A) Representative current recording of CaV1.2 currents recorded on Q-Patch (Sophion). B) CaV1.2 current rundown recorded on Q-Patch. The stability of the current signal was tested, since these cells are known for their run-down. The stability of the CaV1.2 current response was investigated in an assay running for 20. The average current response was -1.6 ± 3.8 nA, $n=32$ and the average run-down was $21.7 \pm 3.8\%$ over 20 min ($1.1 \pm 0.2\%$ per minute), $n=32$. Data kindly provided by Sophion

2 VALIDATION OF HEK-293 CA_v1.2 CELLS

2.1 Biophysical Validation

Ca_v1.2 currents were measured by means of the patch-clamp technique in the whole-cell configuration. The bath solution contained (in mM) NaCl 100, KCl 4, NMDG 40, CaCl₂ 5, MgCl₂ 1, HEPES 10, Glucose 5 and Sorbitol 5. The pH was adjusted to 7.40 with HCl. The pipette solution consisted of (in mM) Cs Methansulfonate 108, MgCl₂ 4.5, CaCl₂ 1, Phosphocreatine Na₂ 5, Creatine 5, Pyruvate 5, Oxalacetate 5, Na₂ATP 4, HEPES 24, EGTA 10. The pH was adjusted to 7.20 with CsOH. A pipette resistance of 3.5 to 4 MΩ was used to seal the cell. After formation of a GΩ seal between the patch electrodes and individual Ca_v1.2 stably transfected HEK-293 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, inward calcium currents were measured upon depolarization of the cell membrane from a holding potential of -80 mV to +50 mV in 10 mV increments of 1000 ms duration (Fig. 1). These pulses were followed by a pulse to +10 mV (300 ms) to record the tail current amplitude. The voltage pulses were run at intervals of 10 s.

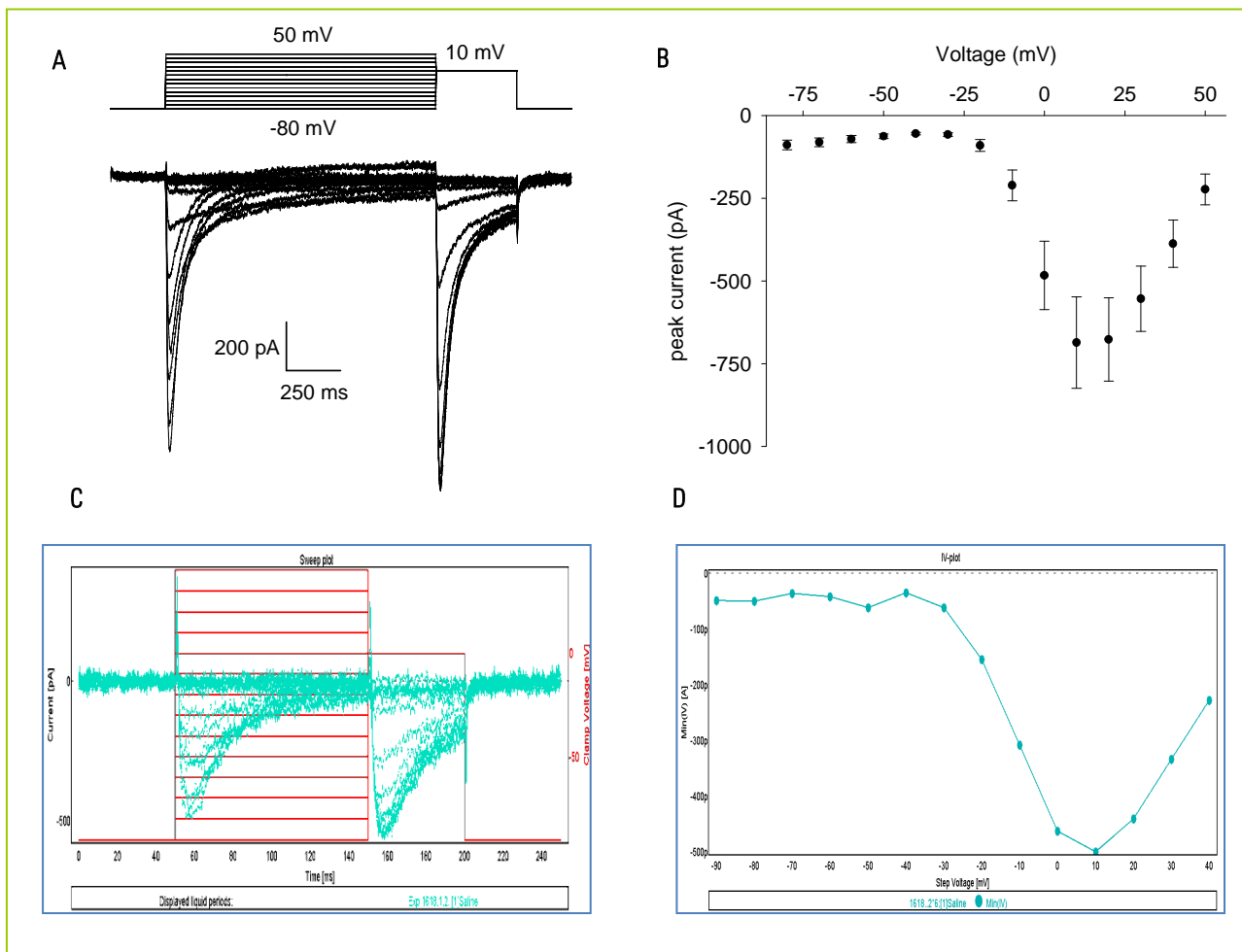


Fig. 2: A) Representative current recording of Ca_v1.2 currents (manual patch-clamping). B) Ca_v1.2 IV curve elicited upon depolarization of the cell membrane from holding potential (-80 mV) up to +50 mV in 10 mV increments. C) Representative current recording of Ca_v1.2 currents (automated patch-clamping). D) Ca_v1.2 IV curve elicited upon depolarization of the cell membrane from holding potential (-90 mV) up to +40 mV in 10 mV increments recorded with manual patch-clamping.

The minimum of the IV curve was found at +20 mV (manual patch clamping) or 12.1 ± 1.0 mV, $n=78$ (automated patch-clamping). Based on the IV curve the activation curve was constructed. The $V_{0.5}$ value was determined as 1.34 ± 1.88 mV ($k=6.50 \pm 0.66$), $n=6$ (Figure 3).

To obtain the inactivation curve Cav1.2 cells in the whole-cell configuration were depolarized to potentials between -80 mV and +50 mV for 1 s, followed by a test pulse to +10 mV (300 ms). The peak currents were plotted versus the voltage of the depolarizing pre-pulse and fitted with a Boltzmann equation. The $V_{0.5}$ value was determined as -16.40 ± 1.79 mV ($k=7.56 \pm 0.30$), $n=6$. Stimulation frequency: 0.1 Hz (Figure 3).

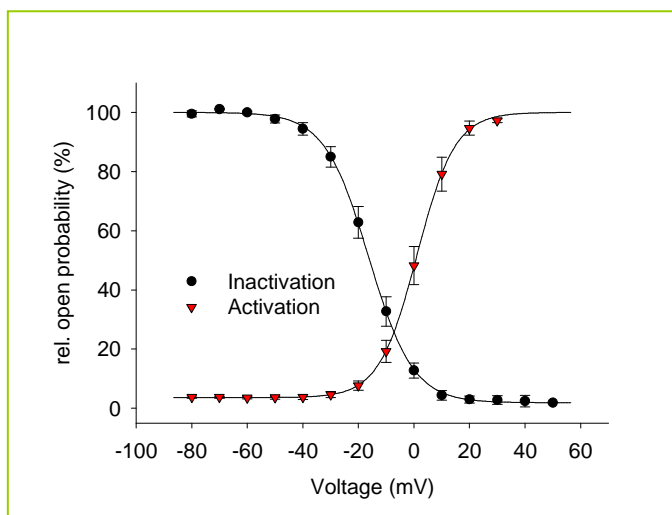


Fig. 3: A) Cav1.2 activation curve obtained from the IV curve by dividing the current amplitudes by the applied voltage minus the reversal potential. B) Inactivation curve of Cav1.2 currents after depolarization for 1 s and a repolarising pulse to 10 mV (100 ms).

2.2 Pharmacological Validation

Cav1.2 cells were used in the whole-cell configuration. The effect of known Ca^{2+} channel blockers was tested on these cells. After the current amplitude had stabilized increasing concentrations of the test substances were perfused. Cumulative dose response curves were generated and the IC_{50} values determined. Between two voltage pulses, the cells were clamped to -80 mV. After a pre-pulse of -50 mV (100 ms) the cells were clamped to 0 mV for 200 ms to open the Cav1.2 channels. The time course of the peak current amplitudes were analyzed, normalized to the current amplitude under control conditions and the effect of each tested concentration calculated.

The results are summarized below:

Table 1: IC_{50} values

Compound	IC_{50} value	Hill coefficient
Nifedipine	78.44 nM	0.80
Isradipine	5.99 nM	0.93
Verapamil	8.15 μ M	0.74

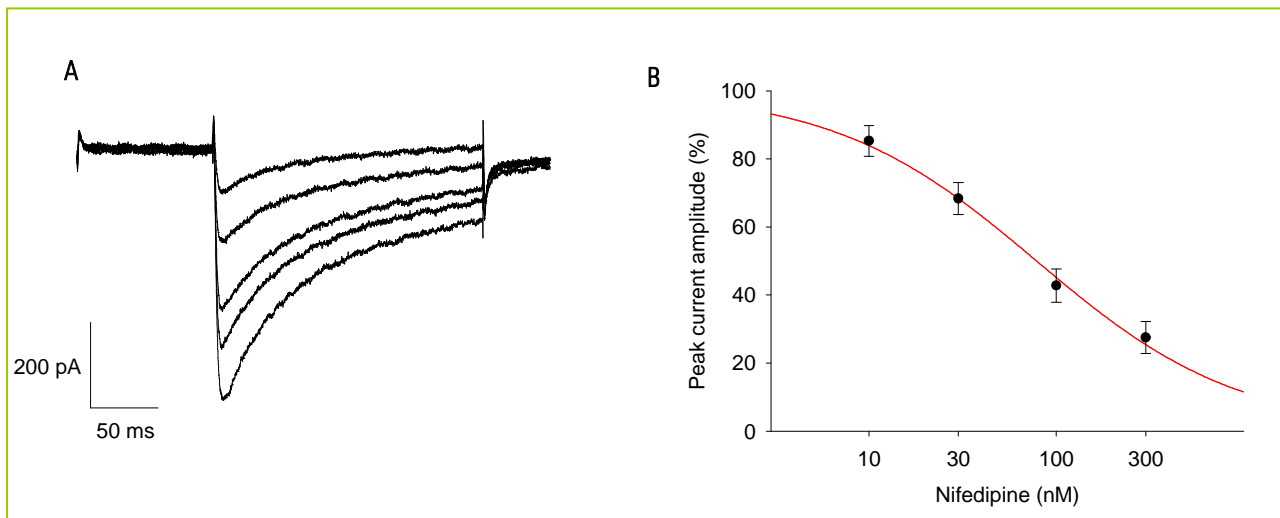


Fig. 5: Block of CaV1.2 by Nifedipine A) Representative current recording. 0 nM, 10 nM, 30 nM and 100 nM were perfused in increasing concentrations. B) Dose response curve of CaV1.2 currents blocked by Nifedipine. The IC₅₀ was determined to be 78.44 nM (Hill coefficient: 0.80).

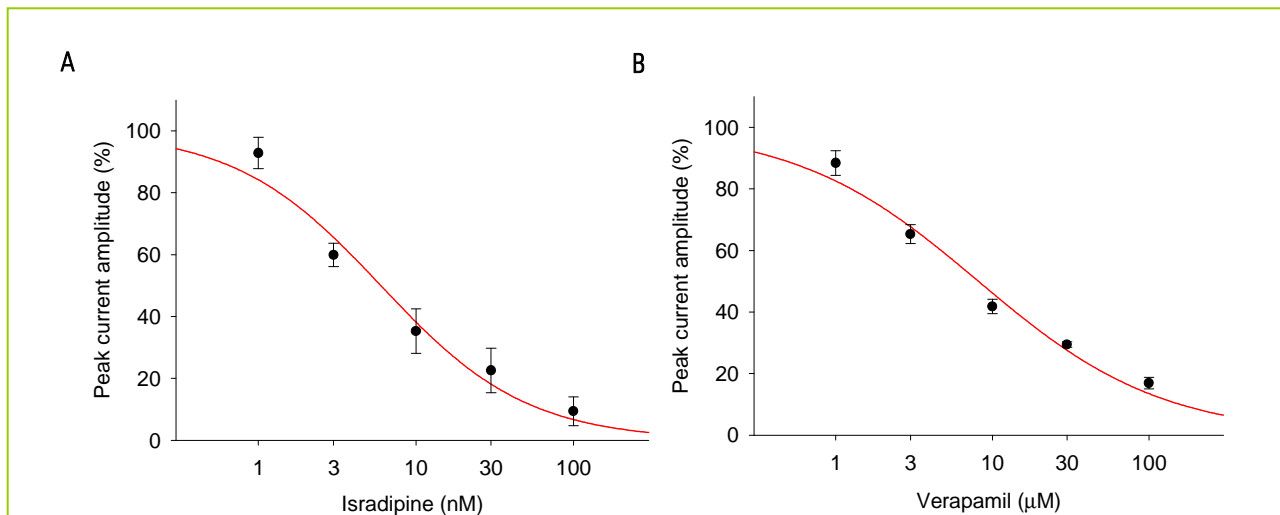


Fig. 4: A) Dose response curve of CaV1.2 currents blocked by Isradipine. The IC₅₀ was determined to be 5.99 nM (Hill coefficient: 0.93) B) Dose response curve of CaV1.2 currents blocked by Verapamil. The IC₅₀ was determined to be 8.15 μM (Hill coefficient: 0.74).

2.3 CiPA Stimulation Protocols

In Autumn 2015 the CiPA (Comprehensive In Vitro Proarrhythmia Assay) consortium suggested two voltage protocols to test drug effects on Cav1.2 channels using patch-clamping. The B'SYS' Cav1.2 cell line is a suitable test system for the CiPA.

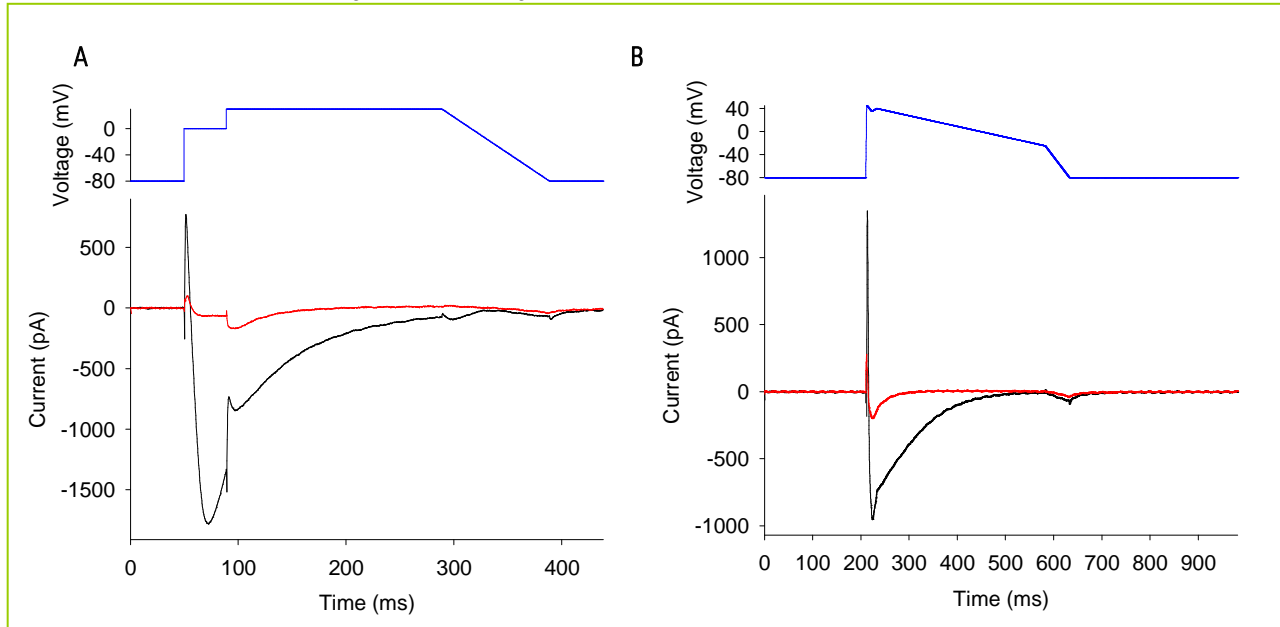


Fig. 6: B'SYS Cav1.2 HEK 203 under CiPA voltage protocols: Black: Control conditions, red: 100 μ M Verapamil, blue: voltage protocol A) Cav1.2 voltage protocol B) Action potential similar stimulation protocol.

2.4 Fluorescence Validation

For the validation of a Fluorescence Screening the Flexstation III of Molecular Devices in combination with the FLIPR 6 Calcium Assay Kit from Molecular Devices was used.

The effects of different K^+ concentrations to induce a Calcium influx into the cells were determined:

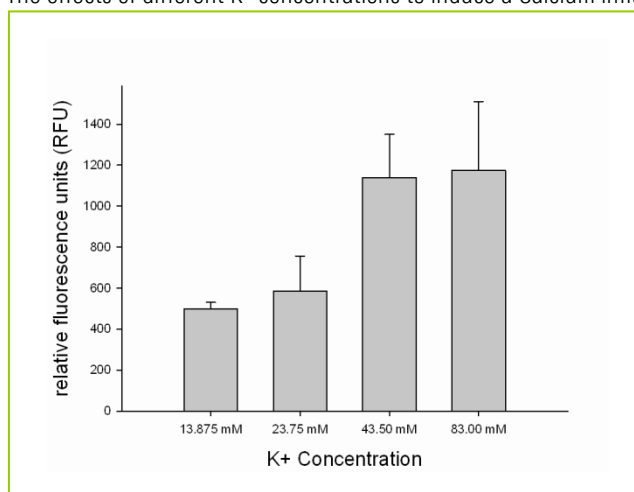


Fig. 7: Effects of different K^+ concentrations on relative fluorescence unit measured. The highest tested concentration of 83.00 mM K^+ was used for the following experiments.

The effects of known Ca^{2+} channel blockers were tested with an in-well concentration of 83 mM K^+ .

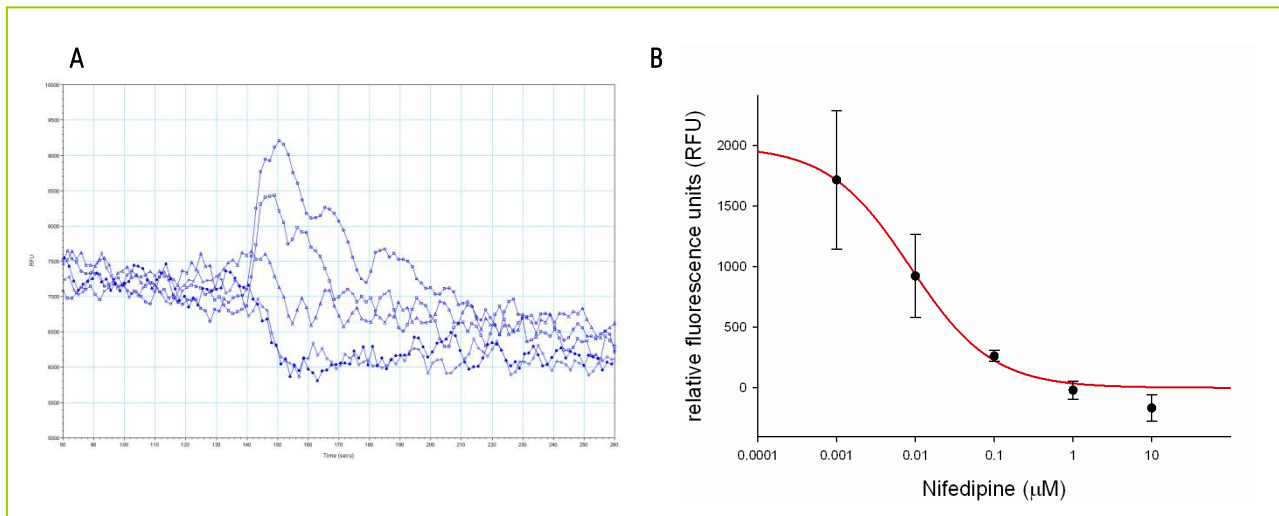


Fig. 8: A) Representative fluorescence traces of $\text{Ca}_v1.2$ currents blocked by Nifedipine. B) Dose response curve of $\text{Ca}_v1.2$ currents blocked by Nifedipine. The IC_{50} was determined to be 8.60 nM (Hill coefficient: 0.84).

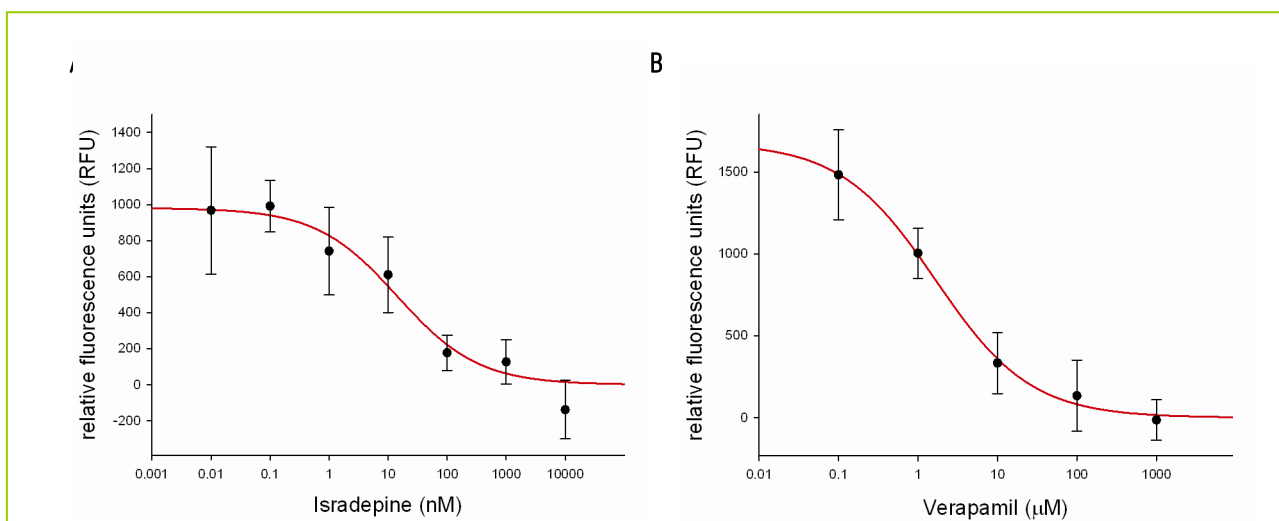


Fig. 9: A) Dose response curve of $\text{Ca}_v1.2$ currents blocked by Isradipine. The IC_{50} was determined to be 14.34 nM (Hill coefficient: 0.63) B) Dose response curve of $\text{Ca}_v1.2$ currents blocked by Verapamil. The IC_{50} was determined to be 1.67 μM (Hill coefficient: 0.73).

The results are summarized below

Table 2: IC_{50} values Fluorescence

Compound	IC_{50} value	Hill coefficient
Nifedipine	8.60 nM	0.84
Isradipine	14.34 nM	0.63
Verapamil	1.67 μM	0.73

3 CELL CULTURE CONDITIONS

3.1 General

HEK-293 Cav1.2/ α 2 δ 1/ β 2 cells are incubated at 37°C in a humidified atmosphere with 5% CO₂ (rel. humidity > 95%). The cells are continuously maintained and passaged in sterile culture flasks containing DMEM/F12 medium supplemented with 10% foetal bovine serum, 1.0% Penicillin/Streptomycin solution and 100 µg/mL Hygromycin B, 15 µg/mL Blastcidin and, 0.4 µg/mL Puromycin. The HEK-293 Cav1.2 cells are passaged at a confluence of about 80%.

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% confluence at 1:3 to 1:5 ratio.

3.2 Recommended Complete Medium

500 mL DMEM/F12 with L-Glutamine or GlutaMax I

10% FBS

1.0% Penicillin/Streptomycin

3.3 Antibiotics

HEK-293 Cav1.2 clones were selected under 250 µg/mL Hygromycin B, 15 µg/mL Blastcidin, and 0.6 µg/mL Puromycin antibiotic pressure.

To cultivate HEK-293 Cav1.2 cells, also a reduced antibiotic pressure (100 µg/mL Hygromycin B, 15 µg/mL Blastcidin, and 0.4 µg/mL Puromycin) can be used.

To separate HEK-293 Cav1.2 cells from untransfected cells, use 250 µg/mL Hygromycin B, 15 µg/mL Blastcidin, and 0.6 µg/mL Puromycin.

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

3.4 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-25 culture flask containing 5 mL complete medium.
- Incubate cells at 37°C for 4-6 hours to allow the cells to attach to the bottom of flask.
- Aspirate off the medium and replace with 5 mL complete medium & antibiotics.
- Antibiotics: 100 µg/mL Hygromycin B / 15 µg/mL Blastcidin/ 0.4 µg/mL Puromycin.
- Incubate cells and check them daily until 70% - 80% confluence is reached.

3.5 Splitting Cells

- Remove medium when cells have reached 70% - 80% confluence.
- 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.
- Use remaining suspension for counting the cells.

3.6 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).

3.7 Freezing Cells

- Prepare fresh freezing medium and keep it on ice.
- Cells should have 80% - 90% confluence 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.
- Incubate cells overnight in a polystyrene box at –80°C.
- Transfer cryovial in liquid nitrogen tank for long-term storage the next morning.

3.8 Inducing Expression of Cav1.2

To induce the expression of Cav1.2 receptors 2.5 µg/mL Tetracycline has to be added 1624 h before experimentation (longer incubation is not recommended).

4 CA_v1.2 SEQUENCE

For some subunits codon optimized sequences were used.

4.1 Cav1.2 alpha 1c subunit

MNANAAAGLAPHEHIPTPGAALSQAAIDAARQAKLMGSAGNATISTVSSTQRKRQOYQKPKKQGSTTATRPPRALLCLTL
 KNPIRRACISIVEWKPFEEIIILLTIFANCVALAIYIPFEDDSNATNSNLERVEYLFLLIIFTVEAFLKVIAYGLLFHPNA
 YLRNGWNLLDFIIVVVGFLFSAILEQATKADGANALGGKGAGFDVKALRAFRVLRPLRLVSGVPSLQVVLNSIIKAMVPLL
 HIALLVLFVIIIIYAIIGLELFMGMKHKTCYNQEGIADVPAEDDPSCALETGHGRQCQNGTVCKPGWDGPKHGITNFDNF
 AFAMLTVFQCITMEGWTDVLYWQDAMGYELPWVYFVSLVIFGSFFVLNLVGLVLSGEFSKEREKAKARGDFQKLRKQO
 LEEDLKGYLWITQAEIDIPENEDEGMDEEKPRNMSMPTSETESVNTENVAGGDI EGENCGARLAHRISKSKFSRYWRRW
 NRFCCRKCRAAVKSNVFWLVIIFLVFLNLTITASEHYNQPNWLTEVQDTANKALLALFTAEMLLKMYSLGLQAYFVSLFN
 RFD CFVVC GGILETIIIVETKIMSPLGISVLRVLRLLRIFKITYWNSLSNLVASLLNSVRSIASLLLLLFLFIIIFSLLG
 MQLFGGKFNDEMOTRRSTFDNFPQSLLTVFQILTGEDWNSVMYDGMAYGGPSFPGMLVCIYFIIILFCGNYILLNVFL
 AIAVDNLADAESLTSAQKEEEEEKERKKLARTASPEKKQELVEKPAVGESKEEKIELKSITADGESPPATKINMDDLQPN
 ENEDKSPYPNPETTGEEDDEEPEMPVGP RPRPLSELHLKEKAVMPPEASAFFIFSSNNRFRQLQCHRIVNDTIFTNLILFF
 ILLSSISLAAEDPVQHTSFRNHILFYFDIVFTTIFTIEIALKMTAYGAFLHKG SFCRNYFNILDLLVSVSLISFGIQSS
 AINVVKILRVLRLRPLRAINRAKGLKHVVQCVFAIRTIIGNIVIVTLLQFMFACIGVQLFKGKLYTCSDSKQTEAEC
 KGNITYKYDGEVDHPIIQPRSWENSKFDFDNVLAAMMALFTVSTFEGWPELLYRSIDSHTEDKGIYNYRVEISIFFIYY
 IIIIAFFMMNIFVGFVIVTFQEQQEQEYKNCELDKNQRQCVEYALKARPLRRYIPKNQHQYKVVVYNSTYFEYLMFVLI
 LLNTICLAMQHYGQSCLFKIAMNILNMLFTGLFTVEMILKLI AFKPKGYFSDPWNVDFDLIVIGSIIIDVILSETNPAEHT
 QCSPSMNAEENSRIISITFFRLFRVMRLVKLLSRGEGIRTLTLLWTFIKSFQALPYVALLIVMLFFIYAVIGMQVFGKIALND
 TTEINRNNNFQTFPQAVLLLFRFCATGEAWQDIMLACMPGKKCAPESEPSNSTEGETPCGSSFAVYFIFISFYMLCAFLIIN
 LFAVIMDNFDYLTRDWSILGPHHLEDFKRIWAEYDPEAKGRIKHLDDVVTLLRRIQPPLGFGLKCPHRVACKRLVSMNMP
 LNSDGTVMFNATLFAVRLTALRIKTEGNLEQANEELRAIKKIWKRTSMKLLDKAMKEAVSAASEDDIFRRAGFLFNHVS
 YQSDGRSAFPQTFQTTQRPLHINKAGSSQGDTEPSHEKLV DSTFTPSYSSTGSNANINNANTALGRLPRPAGYPSTVS
 TVEGHGPPLSPAIRVQEVAVKLSNRRERHVP MCEDELELRRD SG SAGTQAHCLLLRKANPSRCHSRESQAAMAGQEETSQD
 ETYEVKMNHDTEACSEPSLLSTEMLSYQDDENRQLTLPEEDKRDIRQSPKRGFLRSASLGRRASFHLECLKRQKDRGGDI
 SQKTVLPLHLVHHQALAVAGLSPLLQRSHSPASFPRPFATPPATPGSRGWPPQPVPTLRLEGVESSEKLNSSFP SIHCGS
 WAETTPGGGSSAARRVRPVS L MVPSQAGAPGRQFHGSASSLVEAVLISEGLGQFAQDPKFI EVTTQELADACDMTIEEM
 ESAADNII LSGGAPQSPNGALLPFVNCRDAGQDRAGGEEDAGCVRARGRPSEELQDSRVVYVSSL

4.2 Cav beta 2 subunit

MLDRRLIAPQTKYIIPGGSADSYTSRPSDSDVSLEEDREAVRREAERQAQAQLEKAKTKPVAFVVRTNVSYSAAHEDDVP
 VPGMAISFEAKDFLHVKEKFNNDWWIGRLVKEGCEIGFIPSPVKLENMRLQHEQRAKQKGFYSSKSGGNSSSSSLGDIVPS
 SRKSTPPSSAIDIDATGLDAEENDIPANHRSPKPSANSVTSPHSKEKRMPPFFKKTEHTPPYDVVPSMRPVVLVGP SLKGY
 EVTDMMQKALFDFLKHREGRISITRVTADISLAKRSVLNPNPSKHAI IERSNTRSSLAEVQSEIERIFELARTLQLVVL D
 ADTINHQAQLSKTSLAPIIVYVKISSPKVLQRLIKSRGKSQAKHLNVQMVAADKLAQCPELFDVILDENQLEDACEHLA
 DYLEAYWKATHPPSSSLPNPLLSRTLATSSLP SPTLASNSQGSQGDQRTDRSAPIRSASQAE EEP SVEPVKKSQHRSSS
 SAPHHNHRSGTSRGLSRQETFDSETQESRDSAYVEPKEDYSHDHVDHYASHRDHNHRDETHGSSDHRHRESRHRSRDVR
 EQDHNECNKQRSRHKSKDRYCEKDG EVISKKRNEAGEWNRDVYIRQ

4.3 Cav alpha 2 / delta 1 subunit

MAAGCLLALTLTLFQSLIIGPSSEEPFSAVTIKSWVDKMQEDLVTLAKTASGVNQLVDIYEKYQDLYTVEPNARQLVE
 IAARDIEKLLSNRSKALVRLALEAEKVQAAHQWREDFASNEVVYNAKDDLDPEKNDSEPGSQRKIPVFI EDANFRQI S
 YQHA AVHIPTDIYEGSTIVLNELNWT SALDEVFKKNREEDPSLLWQVFGSATGLARYYPASPVWDNSRTPNKIDLYDVR
 RPWYIQGAASPKDMLIIVDVSGSVSGLTLKLRISVSEMLETSLD DDFVNVA SFNSNAQDVSCFQHLVQANVRNKKVLKD
 AVNNITAKGITDYKKGFSFAFEQLLNYNVSRANCNKIIMLFTDGGEERAQEIFNKYKDKKVRVFTFSVQGHNYDRGPIQ
 WMACENKGYYYEIPSIGAIRINTQEYLDVLRPMLVLAGDKAKQVQWNTNVDLDALELGLVITGTLVPVFNITGQFENKTNLK

NQLILGVMGVDVSLEDIKRLTPRF^TLCPNGYYFAIDPNGYVLLHPNLQPKPIGVGIPTINLRKRRPNIQNPKSQEPVTLDFLDAELENDIKVEIRNKMIDGESGEKTFRTLVKSQDERYIDKGNRTYTWTVPVNGTDYSLALVLP^TYSFYI^KKAKLEETITQARYSETLKPDNFEESGYTFIAPRDYCN^DLKISDN^NTEFLLNFNEFIDRKT^PNNPSCNADLINRVLLDAGFTNELVQNYWSKQKNIKGVKARFVVT^DGGITRVYPKEAGENWQENPETYEDSFYK^RSLDNDNYVFTAPYFNKSGPGAYESGIMVSKAVEIYIQKLLKPAVVG^IKIDVNSWIEN^FTKTSIRDPCAGPVCDC^KRNSDVMDCVILDDGGFLLMANHDDYTNQIGRFFGEIDPSLMRHLVNI^SVYAFNKS^YDYQSVCEPGAAPKQGAGHRSAYVPSVADILQIGWWATAAAWSILQQFLLSLTFPRLLEAVEMEDDDFTASLSKQSCITEQTQYFFD^NDSKSFSGVLDCGNC^SRIFHGEKLMNTNLI^FIMVESKGTCPCDTRLLIQAEQTS^DGNPNC^DMVKQPRYRKGP^DVCFD^NNVLEDY^TDCGGVSGLNPSLWYIIGIQFLLLWLVSGSTHRL^L

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