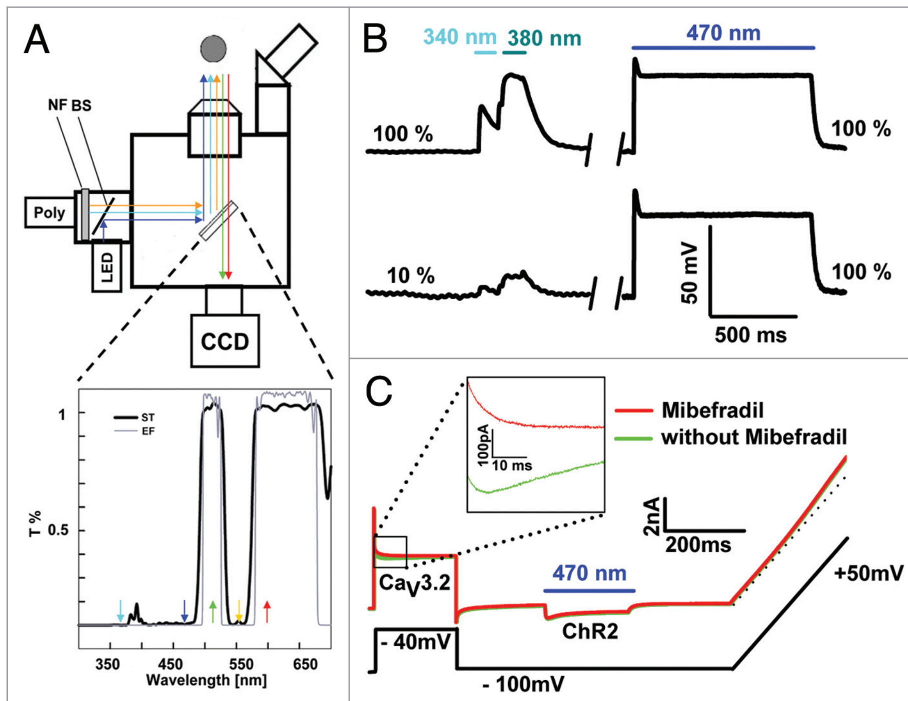

Figure 3. Current-clamp experiment of ChR2 transfected α_{1H}/mTrek cell line at various light intensites. In (A) the instrumental set-up with dual illumination pathway is shown. Illumination with Polychrom V was used for exciting fura-2 (340 nm and 380 nm) and mCherry fluorescence (540 nm). A computer controlled LED light source was used for ChR2 excitation (470 nm). For combining both optical pathways a 70% R/30% T beam splitter (BS) was used. For illumination and detecting the fura-2 and mCherry fluorescence a dual dicroic mirror was used as shown in lower part of (A). In (B) traces at 10% and 100% light intensity for 340/380 nm illumination are shown. At 10% light intensity only small cross-activation of ChR2 at 380 nm is observed. ChR2-illumination at 470 nm was always kept at 100% light intensity, causing a change in membrane voltage of up to 70 mV. In (C) ChR2-mCherry is transiently transfected into the stable α_{1H}/mTrek cell line. A typical voltage-clamp trace showing transient calcium influx upon changing membrane potential from -100 mV to -40 mV. A subsequent illumination with 470 nm at -100 mV cause a ChR2 mediated inward current. Outward rectifying current was observed during voltage ramp from -100 mV to 50 mV, indicating functionality of mTrek. Same voltage protocol was applied in presence of 10 μM mibefradil (*green line*) showing that only $Ca_V3.2$ is inhibited.

assay. A semi-optical approach was used to depolarize the cells optically with 470 nm light and to monitor intracellular calcium changes with fura-2 fluorescence. As hyperpolarisation is necessary for the activation of vast majority of voltage-gated ion channels, the mTrek potassium channel was expressed. Critical depolarisation requires high ChR2 expression levels, high extracellular cation concentration at neutral pH, and about half saturating light intensities at 470 nm. In contrary, hyperpolarisation with K^+ only requires low or moderate mTrek expression.[14] The ion channel under study should be expressed at appropriate levels to get sufficient fluorescence signals at low 340/380 nm light intensities. Under physiological conditions no Ca^{2+} influx through ChR2 was detected with fura-2 during illuminating with blue light. A Ca^{2+} influx was observed only when monovalent cations were substituted with NMG^+ and after illumination longer then 10 s (data not shown). This result is in good agreement with reported values.[8,9]

In general, all rhodopsins show broad absorption spectra. Thus ChR2 stimulation and parallel measurements of fura-2 fluorescence is challenging.[18] Since ChR2 does not absorb light above 550 nm, ion-sensitive dyes with longer wavelength absorption are eligible such as Crimson™ or calcium Orange™ (Invitrogen, Carlsbad, CA). However, the fluorescence responses were too small for our systems, most likely due to a low sensitivity or low dye loading (data not shown). Calcium dyes like Fluo4 are more sensitive than fura-2 but the absorption overlaps with the ChR2 spectrum. Consequently, fura-2 was chosen as intracellular calcium probe. Although fura-2 excitation light (380 nm) at high intensity caused full activation of ChR2, cross-activation could be minimized by reducing the light intensity to 10% (**Fig. 3B**). In addition, mTrek hyperpolarized the membrane voltage to the appropriated level. Under these optimized conditions Ca^{2+} influx through the α_{1H} channel can be triggered with bright blue light and monitored with weak UV-light.

The red-shifted channelrhodopsin-1 of *Volvox carteri* (VChR1) shows a lower absorption in the UV range, which would allow stronger fura-2 excitation and might enable the use of genetically-coded calcium sensors without cross-activation of VChR1.[19] But, due to insufficient VChR1-expression in HEK293T-cells a reliable depolarization with green light was not achieved with VChR1 yet (data not shown).

Since lower excitation light reduces cross-activation of ChR2 on the cost of lower fluorescence signal-to-noise ratio, this ratio was increased by longer exposure times. However, longer exposure times reduce the maximum sampling rate and consequently time resolution. Here, calcium rises appear within less than 1 s, which required a sampling rate of at least 500 ms.

Sampling rate is limited in our system due to incapability to activate ChR2 and measure fura-2 emission simultaneously. Thus, the sampling rate was limited to 8 Hz corresponding to a minimum exposure time of 125 ms with minimum blue light activation of 50 ms for ChR2 (**Figs. 4C and 5B**).

Whereas mibefradil did not inhibit ChR2 and mTrek, a relatively high hill coefficient was obtained (**Fig. 5A**). This is most likely due to long exposure time of 500 ms which averages Ca^{2+} influx to zero.

ChR2 was transiently expressed to establish the ion-channel assay. Thus the degree of depolarization was dependent on ChR2 expression levels. As characterized previously, channelrhodopsins show a relatively linear light-intensity/current profile.[20,21] A stable expression of ChR2 in a host-cell in combination with mTrek will offer more precise and defined voltage control.

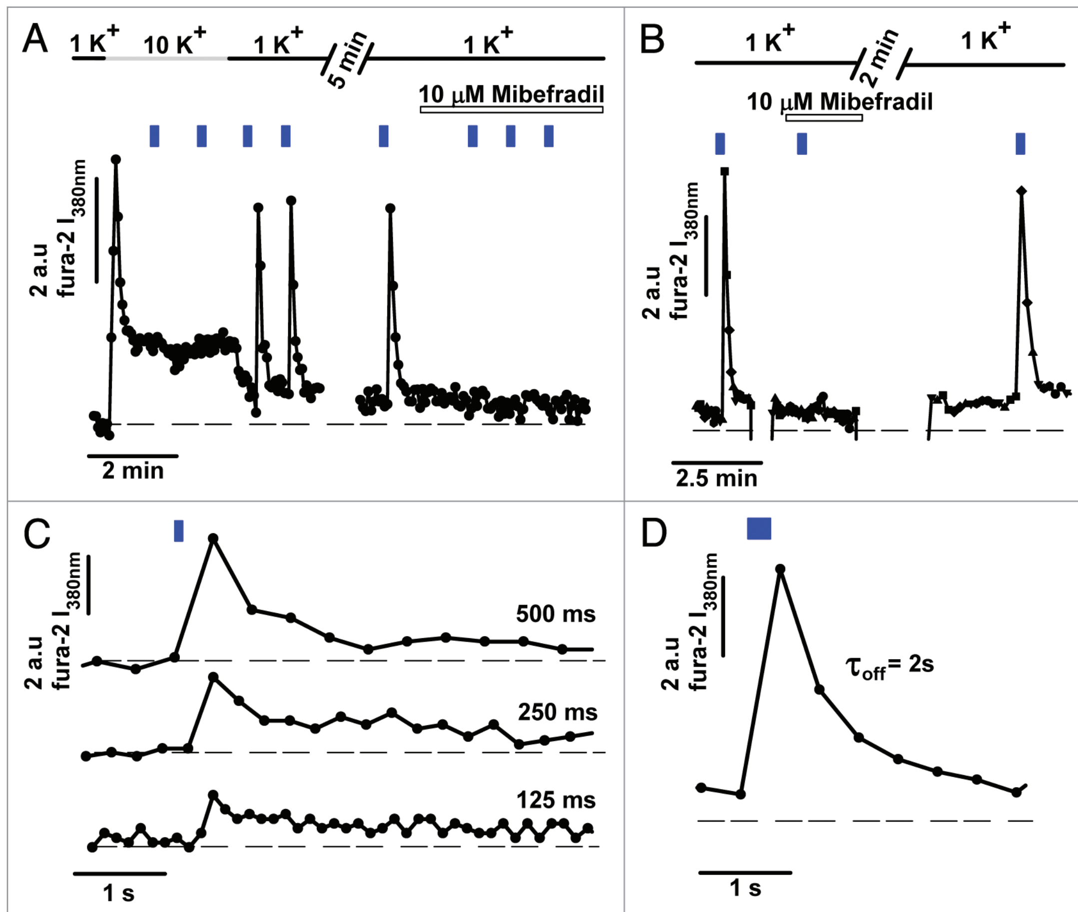

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Figure 4. A ChR2-mCherry expressing HEK-α_{1H}/mTrek cells were loaded with fura-2 and subjected to pulses of blue light in different extracellular buffer conditions. Only when cells are hyperpolarized by a low concentration of K^+, light pulses evoked transient fluorescence increase due to opening of voltage sensitive calcium channel $Ca_V3.2$. Those responses can be evoked repetitively even after a longer pause in same size and kinetic. In (B) a blue ligt pulse evoke calcium influx. Adding 10 μM mibefradile suppresses influx. After a wash-out for more than 3 min, blue light again can evoke calcium influx showing the reversibility of activation. In (C) fluorescence responds from a single cell upon blue light stimulation with different exposure times. Higher sampling rate corresponding too smaller exposure times leads to a smaller signal-to-noise ratio. (D) Shows enlarged, single responds revealing a decay time ~2 s for the fluorescence increase.

Materials and Methods

Cell culture. HEK293 cells stably expressing α_{1H} subunit of $Ca_V3.2$ and mTrek1 were kindly provided by Paula Q. Barrett. Generation and properties of this cell line are described previously.[17]

Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% (w/w) penicillin and streptomycin (Biochrom, Berlin, Germany) at a constant level of 5% CO_2 and 37°C. To maintain the stable expression of both genes, a final concentration of 400 μg/ml G418 and 0.4 μg/ml Puromycin (Sigma-Aldrich, St. Louis, MO) were added to the medium. For measurements 2.5 x 10^5 cells were seeded on a glass cover slip. After 24 h cells were transfected with ChR2-mCherry plasmid using TransPass (NEB, Beverly, MA) and the cultivation was continued for another 24 h. Prior fluorescence or patch-clamp measurements, cells were treated with DME + 10% FBS with 2 μM all-trans retinal.

Patch-clamp measurements. All voltage clamp measurements were performed on an Axiovert 35 (Carl Zeiss Jena, Jena, Germany) with a 40x N.A 1.3 objective. For electrical measurements an Axopatch200B with peltier-cooled headstage (Molecular Devices, Foster City, CA) or EPC-7 (List Electronic, Darmstadt) were used. Analogue data were digitized with Digidata1440 (Molecular Devices, Foster City, CA). For applying protocols for data recording, pClamp software version 10.1 (Molecular Devices, Foster City, CA) was used. Patch pipettes were pulled with micropipette puller model P-97 (Sutter Instrument Co., Novato, CA) from micro-haematocrit-tubes (Hecht-Assistant, Sondheim, Germany). Pipette solution contained [in mM]: 110 NaCl, 10 EGTA, 2 $MgCl_2$, 2 $CaCl_2$, 2 KCl and 10 Hepes. pH was adjusted with NMG/HCl to 7.2 and 280 mOsm. As reference a silver/silver chloride electrode with 1% agarose and 100 mM NaCl cushion was used. External solution contained [in mM]: 140 NaCl, 2 KCl, 2 $MgCl_2$, 2 $CaCl_2$ and 10 Hepes (pH 7.2). All external solution were adjusted to 320 mOsm with glucose and

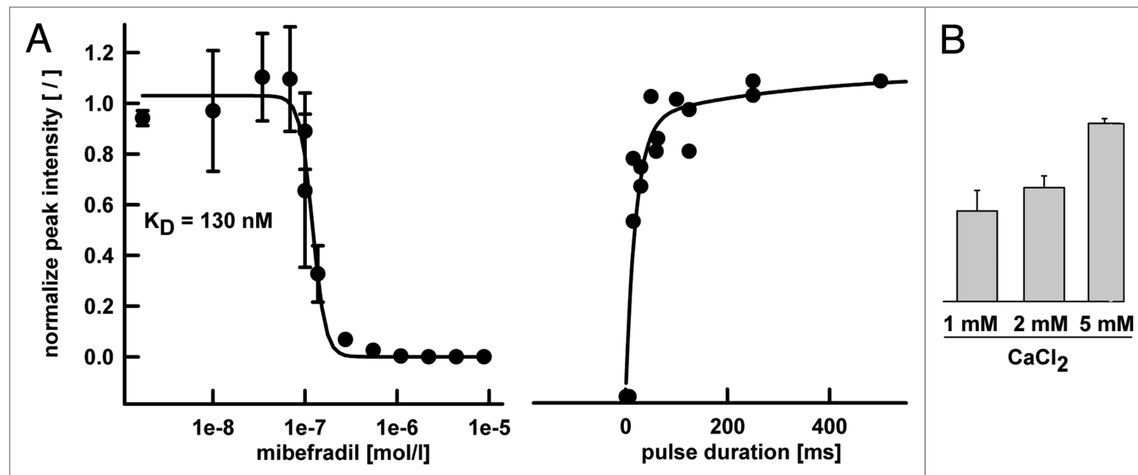

Figure 5. In (A) normalized fluorescence peaks are plotted against the concentration of mibefradile revealing a dose-respond curve with a K_D value of 130 nM. The inset shows that increase peak responses for different concentration of $CaCl_2$. In (B) normalized peak responds on different pulse length of blue light illumination. Pulses longer then 50 ms peaks showed maximum amplitude. All data points are resembled from more then three independent measurements.

filtered with 0.22 μm filter. Under these conditions pipettes had a resistance of 1.5–2 MΩ. White light from a 75 W Xenon lamp (Leistungselektronik, Jena, Germany) was software-controlled by a shutter (Uniblitz, Rochester, NY) and launched into a 1.7 mm light guide (Optran UV N.A. 0.22, CeramOptec GmbH, Bonn, Germany). The light guide was mounted onto back-side of the microscope. Light was filtered by a band interference filter (F49-470, AF-Analysetechnik, Tübingen, Germany) and applied to the specimen via the epiilluminescence port through the objective.

Fluorescence measurements. All fluorescence experiments were performed on a IX70 microscope (Olympus, Tokyo, Japan) with a 40x N.A. and 1.35 Oil objective with high UV transmission. A schematic set-up is shown in **Figure 3C**. Shortly, the light guide of the Polychrome V unit (TILL Photonics, Planegg, Germany) was mounted on the epiilluminescence port. For activation of ChR2 a self-made analogue controlled LED circuit was designed. The LED (5027-PB12, Luxeon, Brantford, CA) was coupled into a 3 mm plastic light guide which was then also mounted into the back side of the microscope and combined with the beam from the polychrome via a 70% R/30% T beam splitter. Combined ChR2- and fura-2-excitation light was passed through the objective via a dualband dichroic mirror (FF493/574, AF-Analysetechnik, Tübingen, Germany). Fura-2 fluorescence was recorded with a CCD Imago camera (TILL Photonics, Planegg, Germany).

Cells were loaded with fura-2-AM solved in DMSO and diluted in DMEM + 10% FBS to a final concentration of 4 μM. Cells were then incubated for 20 min at 37°C with 5% CO_2. Afterwards cells were washed 3 times with [in mM] 140 NaCl, 2 KCl, 2 $MgCl_2$ and 2 $CaCl_2$ and incubated for another 10 minutes. After mounting the cover slip into the measuring chamber and connecting the chamber to the perfusion system, cells were then checked for mCherry and fura-2 fluorescence.

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Data analysis. Data were exported from Tillvision 4.3 Software (TILL Photonics, Planegg, Germany) and analyzed using a self-written MatLab 7.0.1.24704 (R14) program that allows background fluorescence subtraction and analysis of single cells. For fura-2 experiments at high excitation intensities, fura-2 fluorescence ratios at 340 nm/380 nm are graphically presented. For ChR2 experiments with low excitation light intensity, only 380 nm emission was recorded and displayed. Graphs were designed with SigmaPlot (SPSS Science, Chicago, IL).

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