Transported by light: optogenetic control of NCX1

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Abstract

The cardiac Na^+-Ca^{2+} exchanger (NCX1) is one of the key modulators of the cardiomyocytes' Ca^{2+} homeostasis and its reverse-mode has been related to several disorders (e.g. heart failure). It therefore represents an important target for both cardiac safety and drug discovery. Robust high-throughput screening (HTS) assays are required to allow early stage of drug discovery, but the so far available assays are poorly specific and involve complex solution exchange protocols.

Our aim was to develop a new HTS-compatible strategy to trigger the NCX1 reverse-mode activity by the use of optogenetics in HEK-293 cells and to validate this approach in human induced pluripotent stem (iPS) cells-derived cardiomyocytes assays, in order to have a more physiological human background.

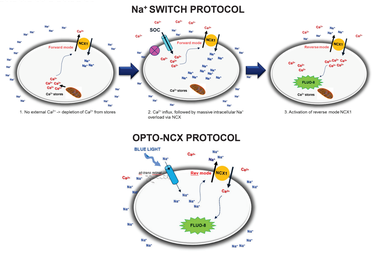
As a proof of concept, we generated a pure HEK-293 clone stably expressing both Channelrhodopsin (ChR2) and NCX1, and we confirmed their functionality with manual patch clamp in voltage-clamp mode and standard fluorescence protocols at FLIPR^{TETRA}, measuring intracellular Ca^{2+} changes or membrane depolarization.

Our rationale is that following the ChR2 light stimulation we promote conditions that favour the reverse-mode function of NCX1, which can be evaluated by monitoring intracellular Ca^{2+}. The same "Opto-NCX1" protocol was used in iPS-derived cardiomyocytes co-cultured with ChR2-expressing HEK-293 cells. The contribution of NCX1 to the global Ca^{2+} cycling of the resulting syncytia was evaluated by pharmacological tools at FLIPR^{TETRA}.

Our results indicate that ChR2 activation by blue light is successful in triggering NCX1 reverse-mode function. This is confirmed by the effect of specific blockers, such as KB-R7943.

Thus, we have developed a new optical tool suitable to run HTS, looking for novel molecules acting on NCX1, and to evaluate potential cardiotoxicity of compound, in the early phase of drug discovery campaigns. This approach can be extended to both patient-specific and heterologous backgrounds.

Rationale



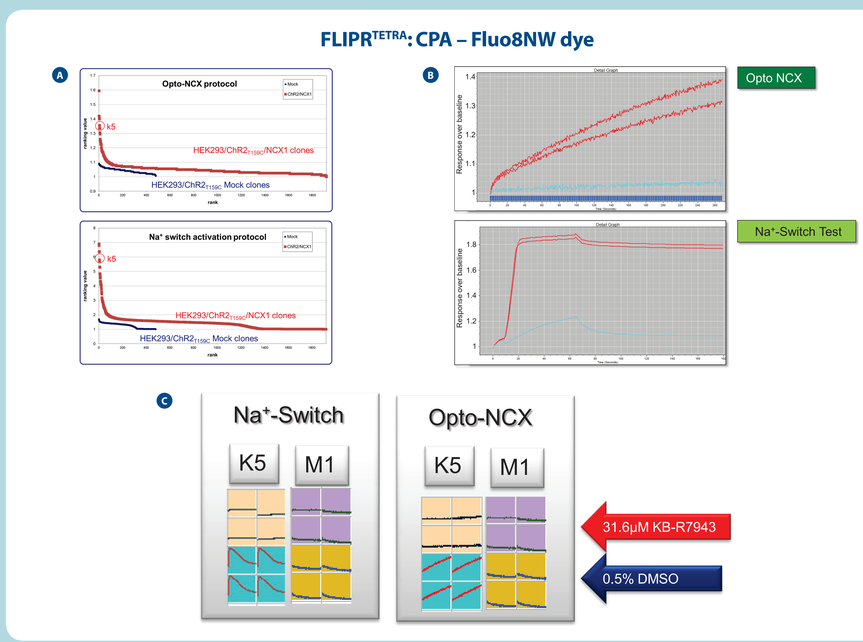
Cell line selection

HEK-293/ChR2 cells were stably transfected with the NCX1 gene or an empty vector (mock). After ten days of antibiotic selection, cells were put in Limiting Dilution and 21 days later a Clone Pool Analysis (CPA) was performed at FLIPR^{TETRA} with a Ca^{2+}-sensitive fluorescent dye (Fluo8-NW) . The presence of NCX1 was assessed using the Na^+ switch and the Opto-NCX protocols (**figure A**, lower and upper panel, respectively).

The figure B illustrates representative FLIPR^{TETRA} traces recorded with both the "Opto-NCX" and the "Na^+ switch" protocols (upper and lower panel, respectively).

The 12 best responding positive clones and 4 mock clones were picked, expanded and retested seeding 15,000 c/w for 24 hrs. The classical "Na^+ switch" and the innovative "Opto-NCX" protocols were used (left and rights panels, respectively).

The figure C shows exemplificative traces for "k5 positive" and "M1 mock" clones (K5 and M1, respectively) incubated in Tyrode's modified buffer containing 0.5% DMSO or in presence of 31.6µM KB-R7943 (lower and higher traces, respectively). The clone k5 was the final selected clone.

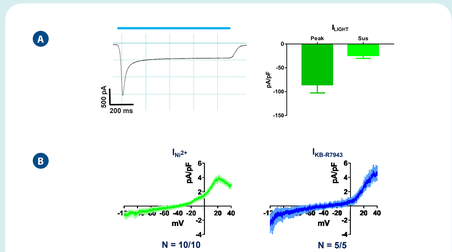


Electrophysiological validation

A. **The figure A** illustrates the "light-induced current" with representative traces and quantitative analysis (left and right panels, respectively). The two components of ChR2$_{T159C}$ currents are indicated as I_{peak} (transient component) and I_{sus} (sustained component).

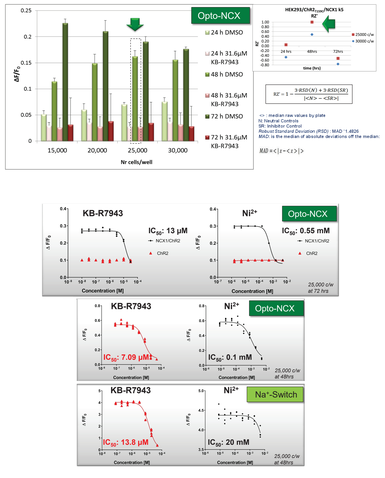
All light-challenged cells showed a response to light (n = 15).

B. **The figure B** shows the average (± confidence intervals) Ni^{2+}- and KB-R7943-sensitive currents evoked by slow voltage ramps: I_{Ni} and I_{KB-R} (left and right panels, respectively). N is shown below the graphs (x/x: cells showing response to blocker/total cells tested)



FLIPR^{TETRA} assay optimization

15,000; 20,000; 25,000 or 30,000 cells/well were seeded in black-clear poly-D-Lys 384 MTP for 24 – 48 – 72 h. FLIPR^{TETRA} functional test was performed using the "Opto-NCX" protocol. NCX1 signal was measured after the injection of 31.6 µM KB-R7943 or 0.5 %DMSO (final concentration) in Tyrode's buffer (exc. 470-495nm/em.515-575). RZ'-prime calculation was performed using as neutral control: 0.5% DMSO and 31.6 µM KB-R7943 as inhibitor control, following the reported formula. The condition 25,000 c/w for 48 h was finally selected. Concentration-response curves of KB-R7943 and Ni^{2+} obtained with the "Opto-NCX" protocol using the HEK-293/ChR2T159C/NCX1 and the HEK-293/ChR2T159C (mock) cell lines or the HEK-293/ChR2T159C/NCX1 cell line alone (upper + middle and lower panels, respectively). IC_{50} calculation was generated with GraphPad Prism 6 software.



Conclusions

In the present work we have generated a pure cell line stably expressing ChR2$_{T159C}$ and NCX1, whose functionality has been assessed through electrophysiology and fluorescence techniques. We have demonstrated that activation of ChR2 by blue light is able to trigger the NCX1 reverse mode activity, that can be directly measured by Ca^{2+} sensitive fluorescent dyes. Therefore, the "Opto-NCX" protocol represent an innovative, robust, simple and reliable strategy for studying the NCX1 transporter in high-throughput screening assays.

SURFE^{2}R analysis

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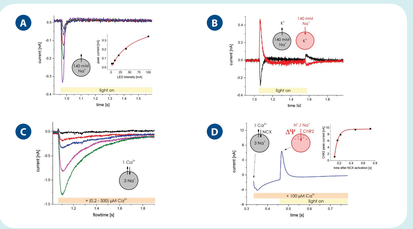

A. ChR2$_{T159C}$ currents induced by blue light of different intensities.
Membrane vesicles were pre-loaded with 140 mM Na^+ and flushed with 140 mM KCl to establish an outward directed Na^+ gradient. Then ChR2 was activated by the application of a 1 s light pulse. Channel opening leads to the efflux of Na^+ which is detected as a fast decaying negative current. The peak current depends on the light intensity. The inset shows the saturation of ChR2$_{T159C}$ peak currents with increasing light intensity.

B. Polarity of ChR2$_{T159C}$ currents depend on the direction of the Na^+ gradient.
Membrane vesicles were pre-loaded either with 140 mM NaCl or KCl (black and red traces, respectively). Then the proteoliposomes were flushed with 140 mM KCl or NaCl (black and red traces, respectively) to generate outward and inward directed Na^+ gradients, respectively. Finally, ChR2 was activated by the application of a 400ms light pulse. Depending on the direction of the Na^+ gradient, Na^+ influx or efflux are observed as positive or negative currents respectively.

C. Ca^{2+} concentration dependence of NCX1 currents
Membrane vesicles were pre-loaded with 140 mM NaCl. After establishing the outward directed Na^+ gradient by flushing with a buffer containing 140 mM KCl, NCX1 was activated by a solution containing different $CaCl_2$ concentrations (0.2 – 500 µM). The currents observed show a negative polarity, reflecting the NCX1 1stoichiometry of 3 Na^+ going out, and 1 Ca^{2+} going in.

D. NCX1 transport activity generates a potential which can be detected by ChR2$_{T159C}$
Membrane vesicles were pre-loaded with 140 mM NaCl. After establishing the outward directed Na^+ gradient by flushing with a buffer containing 140 mM KCl, NCX1 was activated by a solution containing 100 µM $CaCl_2$. During NCX1 transport activity an inside negative potential is generated. A few ms after the raise of the NCX1 current a 1s light pulse was applied to activate ChR2$_{T159C}$. A resulting current of positive polarity is observed, reflecting Na^+ and/or H^+ going in, driven by the inside negative potential which was generated by NCX1.
The inset shows the change of the ChR2$_{T159C}$ peak currents with different time intervals between NCX1 and ChR2$_{T159C}$ activation. The longer the NCX1 activation, the higher the inside negative potential, the more the ChR2$_{T159C}$ signal is affected.

Methods

HEK-293 cells expressing ChR2$_{T159C}$, previously generated in Axxam, were stably transfected with NCX1 gene or with an empty vector.
After ten days of antibiotic selection, cell pools were put in Limiting Dilution at 0.3 cells/well in 10x96 MTP. 21 days later a clone pool analysis (CPA) was performed 24h after replica plate in black-clear poly-D-Lys 384 MTP.
The 12 best responding positive clones and 4 mock clones were picked and expanded. All these clones were retested at FLIPR^{TETRA} as counted cells with both "Na^+ switch" and "Opto-NCX" protocols. The best performing clone was selected and analyzed by manual patch clamp. Further characterization was performed on the final clone by FLIPR^{TETRA} and SURFE^{2}R analysis.

FLIPR^{TETRA} Na^+ switch protocol

25000 cells/well were seeded in poly-D-lysine coated, clear-bottom, black, 384-well polystyrene assay plates. 48 h later, culture medium was removed and cells were incubated with 20 µl/w 0.5X Fluo-8 NW dye in Solution 1 for 45 min at room temperature. Then, cells were incubated for 20 minutes in Solution 2, containing different concentrations of antagonists; 0.1% DMSO was used as vehicle. The activation of the NCX1 in reverse mode was finally obtained injecting Solution 3.

FLIPR^{TETRA} Opto-NCX protocol

25000 cells/well were seeded in poly-D-lysine coated, clear-bottom, black, 384-well polystyrene assay plates. 48 h later, culture medium was removed and cells were incubated with 20 µl/w 0.5X Fluo-8 NW dye for 45 min at room temperature. Different concentrations of antagonists were added and incubated for 20 min at 37°C. The activation of the NCX1 in reverse mode is obtained directly at FLIPR^{TETRA} exciting the cells at 470-495nm for 270 sec every 0.55 sec (exc. 470-495nm/em.515-575).

Electrophysiology

24 hours before experiments, cells were seeded onto poly-D-lysine coated glasses and placed in six-well plates in antibiotic-free medium containing 2 μM all-trans-retinal. Standard whole-cell voltage clamp experiments were performed at room temperature.
To elicit ChR2 currents, cells were continuously kept at -80 mV and blue light stimulation was delivered by a fluorescent lamp. To isolate NCX1 currents, cells were kept at -80 mV and a slow (1 s) voltage ramp from -120 to +40 mV was applied at 1 Hz; NCX1 was blocked applying 5 mM Ni^{2+} or 10 μM KB-R7943. Data was acquired with HEKA EPC10 digitally controlled amplifier in combination with PATCHMASTER software (HEKA Electronics). The NCX1 currents were defined as the Ni^{2+} and KBR-sensitive currents, obtained by digital subtraction.

SURFE^{2}R

HEK-293/ChR2$_{T159C}$/NCX1 cells were cultivated, harvested, lysed and the plasma membrane was collected by partial centrifugation.
The resulting membrane vesicles were adhered to a solid supported lipid membrane (SSM) of 3mm diameter. The ion currents through the vesicle membrane, triggered by sequential perfusion with different buffers, were measured with the SURFE^{2}R N1 device by capacitive coupling.

Solutions

Na+ Switch Protocol

Solution 1 (mM): 130 NaCl, 5 KCl, 1 $MgCl_2$, 5 $NaHCO_3$, 20 HEPES, pH 7.4
Solution 2 (mM): 3 $CaCl_2$, 130 NaCl, 5 KCl, 1 $MgCl_2$, 5 $NaHCO_3$, 20 HEPES, pH 7.4
Solution 3 (mM): 4.5 $CaCl_2$, 130 sucrose, 5 KCl, 1 $MgCl_2$, 5 NaHCO3, 20 HEPES, pH 7.4

Opto-NCX Protocol

Solution 1 (mM): 130 NaCl, 5KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 20 HEPES; 5 µM all-trans retinal; pH 7.4

Electrophysiology

Extracellular solution (mM): 145 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 glucose, pH 7.4
Intracellular solution (mM): 120 KCl, 5.374 $CaCl_2$, 1.75 $MgCl_2$, 10 HEPES, 10 EGTA, 5 Na_2ATP, pH 7.2

SURFE^{2}R

All buffers contain 30 mM HEPES (pH 7.4, NMDG) and 4 mM $MgCl_2$. For the experiments the proteoliposomes were equilibrated in a buffer either containing 140 mM NaCl or
140 mM KCl. A buffer containing 140 mM KCl or 140 mM NaCl was used during solution exchange to establish an outward or inward directed Na^+ gradient respectively. For the activation of NCX a buffer containing 140 mM KCl and different concentrations of $CaCl_2$ were used.

Chemicals

Stock solution of $NiSO_4$ was dissolved 1 M in water; KB-R7943 was dissolved at 100 mM in 100% DMSO.
The final concentration of DMSO was 0.5% for FLIPR^{TETRA}, and 0.1% for manual patch clamp experiments.

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