Transported by light: optogenetic control of NCX1

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Abstract

The cardiac Na+-Ca2+ exchanger (NCX1) is one of the key modulators of the cardiac action potential, and its dysregulation has been related to several cardiovascular diseases (e.g. heart failure). Therefore, research efforts have been focused on the development of drugs that can block NCX1, whose function is essential for Na+ influx or Ca2+ efflux. Although it has been appreciated that Ca2+ influx is a key driver of cardiotoxicity, very little is known about the role of Na+ influx in the early phase of drug discovery, looking for novel molecules acting on NCX1, and to evaluate potential cardiotoxicity of compound, in the early phase of drug discovery. The aim of this study was to develop a new HTS-compatible strategy to trigger the NCX1 reverse mode activity in HEK-293 cells and to validate this approach in human induced pluripotent stem (iPS) cells derived from cardiomyocytes 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 protocol at FLIPR®, measuring intracellular Ca2+ changes or membrane depolarization.

Our rationale is that following the ChR2 light stimulation we promote conditions that favor the reverse-mode function of NCX1. We also developed a novel “Na+-Switch” protocol, using a new Ca2+ sensitive fluorescent dye (FLIPR® Teta-Fluo). The presence of NCX1 was assessed using the “Na+-Switch” and the Opto-NCX protocols (figure 1, lower and upper panels, respectively). The best performing clone was selected by monitoring intracellular Ca2+ changes or membrane depolarization.

The “Na+-Switch” protocol was used in IPS-derived cardiomyocytes co-cultured with ChR2-expressing HEK-293 cells. The contribution of NCX1 to the global Ca2+ cycling of the resulting systolic gap was evaluated by pharmacological tools.

Our results indicate that ChR2 activation by blue light is successful in triggering NCX1 reverse-mode function. This is confirmed and extended to specific blockers, such as KB-R7943, thus, we have developed a new optical tool suitable for 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 other specific and heterogeneous backgrounds.

Rationale

Methods

HEK-293 cells expressing ChR2 (and previously generated in Axxam) were stably transfected with NCX1 gene or an empty vector. After ten days of selective selection, cells were plated in limiting dilution (1 cell/well) and grown in 96-well plates in DMEM supplemented with 5% FCS. Cells were replated on 24-well plates 24 hours after the second passage, then grown for 24 hours until reaching 70%–80% confluency. After 2 days, clones were picked, expanded and all clones were screened at FLIPR® as uncoated cells with both “Na+-Switch” and Opto-NCX protocols, in order to assess the clonal variability for each clone. Functional characterization was performed on the cell clones selected by FLIPR® and SURFER analysis.

FLIPR® “Na+-Switch” protocol

25000 cells/well were seeded onto poly-D-lysine coated, clear-bottom, black, 384-well polyethylene assay plate. 48 hours culture medium was renewed and cells were collected with 20 µL 0.25% Pluronic F-68 dye at 48 hrs at room temperature. Different concentrations of antagonists were added and incubated for 30 min. The activation of the NCX1 reverse mode was obtained by perfusion with a solution containing 100 µM CaCl2 and 140 mM NaCl (black trace, respectively). Then the cells were stimulated with 140 mM KCl to establish an outward directed Na+ current. The blue light is able to trigger the NCX1 reverse mode activity, that can be directly measured by a Ca2+ sensitive fluorescent dye. Therefore, the “Opto-NCX” protocol represent an innovative, simple and reliable strategy for studying the NCX1 transport in high-throughput screening assays.

FLIPR® assay optimization

100000/200000/350000 cells per well were seeded in black clear poly-D-lysine 384 MTP for 24–48–72 h. FLIPR® fluorescent protocol was performed using the “Opto-NCX” protocol. NCX1 activity was measured after the injection of 13 µM KB-R7943 or 0.1% DMSO (final concentration) in Tyrode’s solution (ess. 470–495/nm vs. 515–570). IC50 of KB-R7943 was measured using a buffer containing 100 µM CaCl2 and 140 mM NaCl or 140 mM KCl or 140 mM NaCl or 140 mM KCl and 30 mM HEPES (pH 7.2). The concentration 25,000 c/w for 48 h was finally selected. Concentration-response curves of KB-R7943 and Ni2+ were obtained with the “Opto-NCX” protocol using the HEK-293/ChR2/NCX1 knock-in cell line (Supplementary file). The final concentration of DMSO was 0.5% for FLIPR® and 0.1% for SURFER analysis.

FLIPR® “Opto-NCX” protocol

25000 cells/well were seeded onto poly-D-lysine coated, clear-bottom, black, 384-well polyethylene assay plate. 48 hours culture medium was renewed and cells were collected with 20 µL 0.25% Pluronic F-68 dye at 48 hrs at room temperature. Different concentrations of antagonists were added and incubated for 30 min. The activation of the NCX1 reverse mode was obtained by perfusion with a solution containing 100 µM CaCl2 and 140 mM NaCl (black trace, respectively). Then the cells were stimulated with 140 mM KCl to establish an outward directed Na+ current. ChR2-expressing HEK-293 cells were co-cultured with NCX1 cells and fluorescence was measured with the SURFACE® N1 device by excitation coupling.

Opto-NCX Protocol

1. Solution 1 (mM): 130 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 EGTA, 5 Na2ATP, pH 7.2

2. Solution 2 (mM): 130 NaCl, 5 KCl, 5 NaCl, 5 Na2ATP, pH 7.4

3. Solution 3 (mM): 130 NaCl, 5 KCl, 1 NaCl, 1 MgCl2, 5 Na2ATP, pH 7.4

4. Solution 4 (mM): 130 NaCl, 0 NaCl, 5 KCl, 1 MgCl2, 5 Na2ATP, pH 7.4

Electrophysiology

24 hours before experiments, cells were seeded onto poly-D-lysine coated and placed in standard plates in antibiotic-free medium containing 10% fetal bovine serum to reach 80% confluence. On the day of the experiment all cells were washed twice with a solution containing 2 mM CaCl2 and 140 mM NaCl. The extracellular solution (mM): 145 NaCl, 4 KCl, 1 MgCl2, 10 HEPES, pH 7.2. The final concentration of DMSO was 0.5% for FLIPR® and 0.1% for SURFER analysis.

FLIPR® “Na+-Switch” protocol

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Conclusions

In the present work we have generated a pure cell line stably expressing both Channelrhodopsin (ChR2) and NCX1, whose function is essential for Na+ influx or Ca2+ efflux. Although it has been appreciated that Ca2+ influx is a key driver of cardiotoxicity, very little is known about the role of Na+ influx in the early phase of drug discovery, 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 other specific and heterogeneous backgrounds.

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