Label-free analysis of Na^+/Ca^{2+}-exchanger (NCX) isolated from iPSC-derived cardiomyocytes

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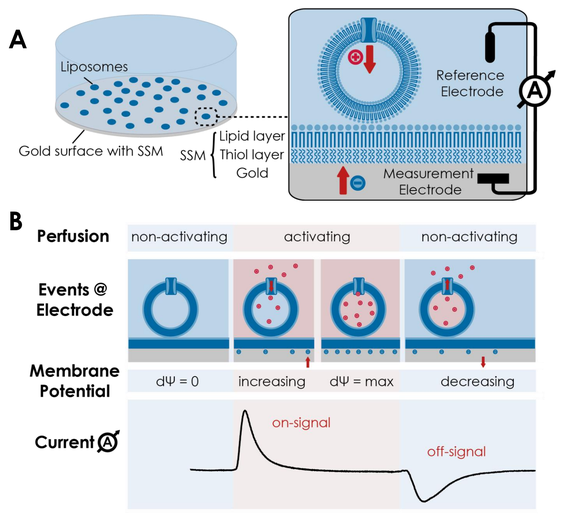
Abstract

The Sodium-Calcium Exchangers (NCX) play an important role in the cellular calcium homeostasis under physiological and pathological conditions. NCX has been of interest as a pharmacological target for many years, in particular because clinical trials involving inhibitors of the sodium-proton exchanger, NHE, have delivered mixed results. Inhibition of the reversed mode of NCX is thought to be beneficial in ischemia/reperfusion injury by reducing cardiac, neuronal and renal infarct areas. Moreover, inhibition of NCX has been proposed to exhibit an anti-arrhythmic effect and therefore, may provide a novel target for the treatment of a variety of arrhythmic pathologies. So far, a number of studies have shown promising results but investigations are limited by the currently available NCX inhibitors such as KB-R7943, SEA-0400 and SN-6 which are only partially specific.

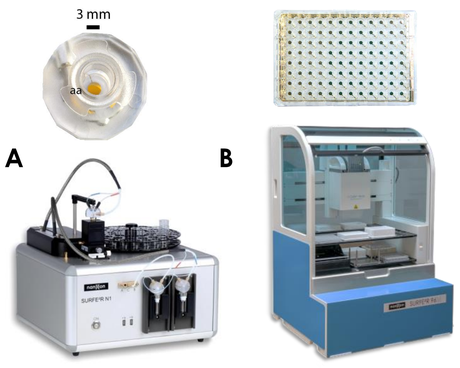
To drive the progress in pharmacological NCX research, new methods to measure NCX function are needed. At the current time, functional investigation of NCX range from patch-clamp, calcium flux assays, Langendorff-perfused hearts to studies in whole animals. We have developed an electrophysiological method to investigate NCX function which is based on the solid supported membrane (SSM) technology. HEK cells overexpressing NCX or human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) were used on a single-well or 96-well SSM electrophysiology device and NCX was recorded from these cells. NCX was activated using Ca^{2+} in the buffer and inhibited by Cd^{2+} and other compounds.

Resolving low amplitude ion currents



"Solid Supported Membrane (SSM)-based electrophysiology" is a method, which allows the resolution of low amplitude electric events in biological membranes, bringing the advantages of electrophysiology to the transporter field. It enables real-time activity measurement of electrogenic SLC-transporters, MFS-transporters and ion pumps, localized in the plasma membrane, in intracellular or bacterial membranes.
Unlike cell-based electrophysiology, a large capacitive sensor coated with a high amount of membrane vesicles or proteoliposomes is used. In this way any process that generates an electrical potential can be registered with high amplification. The method was established in the 90's and has only recently been scaled-up to a 96-well format.

A: Structure of a SSM sensor. The 3 mm diameter thin layer gold electrode is coated with a SSM consisting of a thiol and a lipid monolayer. On top liposomes or membrane vesicles containing the target protein are adsorbed.
B: Protocol of one activation cycle. The sensor is perfused with a passive non activating solution followed by an activating solution, containing a transported substrate or ATP. During this step a transient current occurs, since the active transporter moves charge into the vesicles and this generates a membrane potential.



Instrumentation: We used the SURFE^{2}R N1 **(A)** and the SURFE^{2}R 96SE **(B)** instruments in this project. The respective SSM sensors are depicted above the instruments. The SURFE^{2}R N1 is a flexible single channel measurement instrument, whereas the SURFE^{2}R 96SE is a robotic device designed for experiments that require higher throughput. **C:** Membrane proteins successfully studied using SSM-based electrophysiology

C

Transporters			
Inorganic ions	**Amino acids**	**Sugars**	**Organic ions**
NhaA	PEPT1 (Slc15a1)	SGLT1/2 (Slc5a1/a2)	OCT2 (Slc22a2)
NhaP	YdgR	MelB	CNT1 (Slc28a1)
NhaB	YhiP/DtpB	LacY	ANT (Slc25a4)
NCX1 (Slc8a1)	PutP	FucP	AAC
Clc-7	GltP	XylE	GAT1 (Slc6a1)
EcClc	EAAC1 (Slc1a1)	GlcP	BetP
NirC	PAT1 (Slc36a1)		CHT (Slc5a7)
Amt1-3	ArcD		NupC
AmtB	CAT2B (Slc7a2)		NacT (Slc13a5)
SulP	GlyT1/2 (Slc6a9/a5)		
NIS (Slc5a5)			
NaPi2b (Slc34a2)			
MntH2			

ATPases	Redox-driven ion pumps
NaK-ATPase	Complex I
HK-ATPase	respiratory chain complex I/III
SERCA	respiratory chain complex II/III
V-ATPase	cyctochrome c-oxidase
FoF1-ATPase	respiratory chain complexes I/III/V
Kdp-ATPase	
CopA	**Light-driven ion pumps**
ATP7A/B	Bacteriorhodopsin (BR)
VrPPase	Oxyrrhis marina Rhodopsin
	Rhodopsin-2 (KR2)
	Halorhodopsin (HR)
	Acerhodopsin
	Channelrhodopsin (ChR)

Channels and Pores
Gramicidine
P2X2
nAChR
A/M2
UCP1 (Slc25a7)
TRPC5
TRPA1
CFTR
AQP6

Summary and Future prospects

We have developed two novel methodic approaches for the functional investigation of NCX and have recorded NCX from HEK cells and hiPSCs. NCX was activated by Ca^{2+} in the buffer and could be inhibited by a number of pharmacological agents.

Future Experiments

- Further validation and optimization of the methods is required.
- We intend to develop a third complementary method using a MEA-like system to investigate the influence of NCX inhibitors and cross reactions of NCX inhibitors and pro-arrhythmic drugs on the beating behavior of iPSC derived cardiomyocytes.
- The developed methods shall be applied in a project to characterize drug effects on NCX in more detail.
- The next step intended is to screen the proarrhythmic drugs of the CiPA initiative compound list using the high throughput method for effects on NCX.

Contact us if you are interested in collaborating with us to:

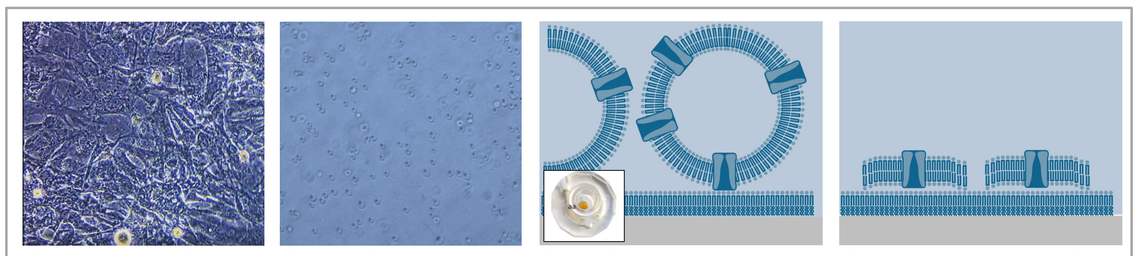
- Apply the high throughput method for the development of novel NCX inhibitors.
- Evaluate the need for NCX safety testing of new drug candidates using the cardiomyocyte based method.

Literature

- Amran et al., Cardiovascular Drug Reviews, Vol. 21, No. 4, pp. 255–276
- Antoons et al., Pharmacology & Therapeutics 134 (2012) 26–42
- Herrmann et al., Cardiovascular Research (2013) 99, 780–788
- Huag, JPTM 87 (2017) 11–23
- Lee and Hryshko, Cardiovascular Drug Reviews, Vol. 22, No. 4, pp. 334–347
- Pignataro, Translational Stroke Research, DOI 10.1007/s12975-013-0308-8
- Watanabe, et al., J. Pharmacol.Sci.102, 7 – 16 (2006)
- Matsuda, et al., 2001. JPET. 298(1): 249-256
- Watanabe & Kimura., 2001. Jpn. J. Pharmacol. 85: 370-375

Measuring NCX activity in human iPSC derived cardiomyocytes

Human iPSC derived cardiomyocytes are being investigated as a model for cardiac safety assessment. To measure native NCX in these cardiomyocytes a cell based assay was developed. Cardiomyocytes were detached from the culture dish and added to the lipid coated SSM sensor. Where the cell connected with the lipid layer. Upon rinsing of the sensor with high fluidic speed, the cells detached again, but a sheet of the cell membrane remains on the sensor. NCX currents can be evoked in these sheets. For a higher NCX signal female cardiomyocytes were used. This method enables the efficient investigation of the isolated cardiac NCX current in a native membrane.



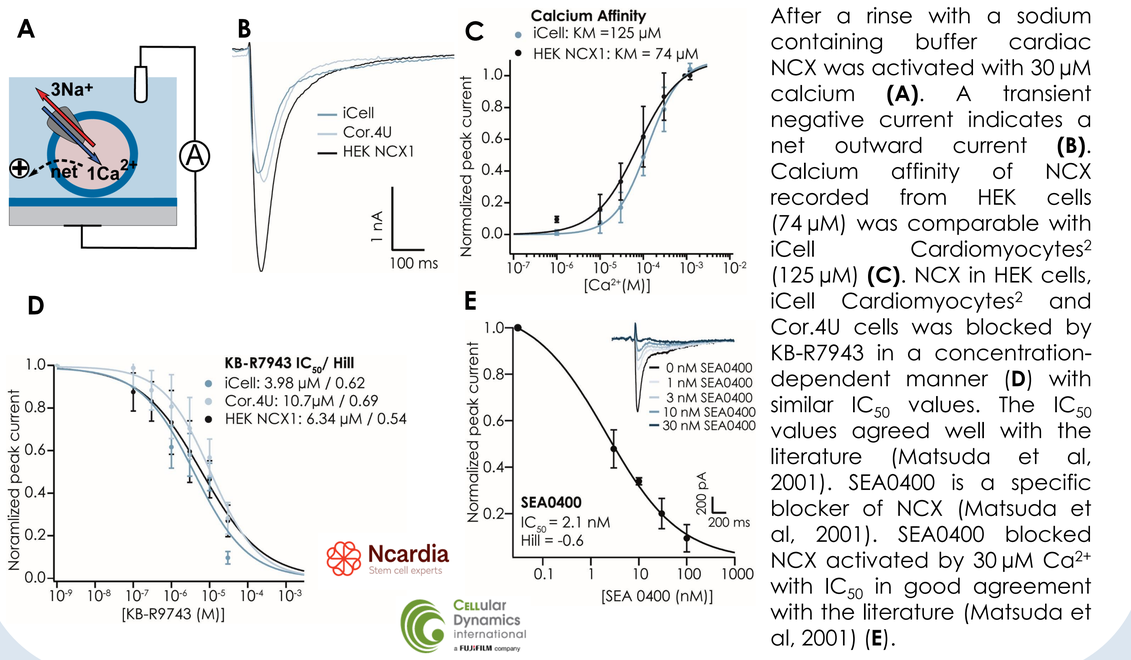
Cor.4U (Ncardia) iPSC derived cardiomyocytes growing adherent on a cell culture dish.

The cardiomyocytes were detached using trypsin

The cardiomyocytes were added to a lipid coated SSM sensor. The sensor was centrifuged to form a tight connection of the cells with the lipid layer

The sensor was rinsed in a fluidic system with high speed. This leads to tearing of the cells – only a membrane sheet remains. This is a working hypothesis, further investigation is necessary.

Sample preparation



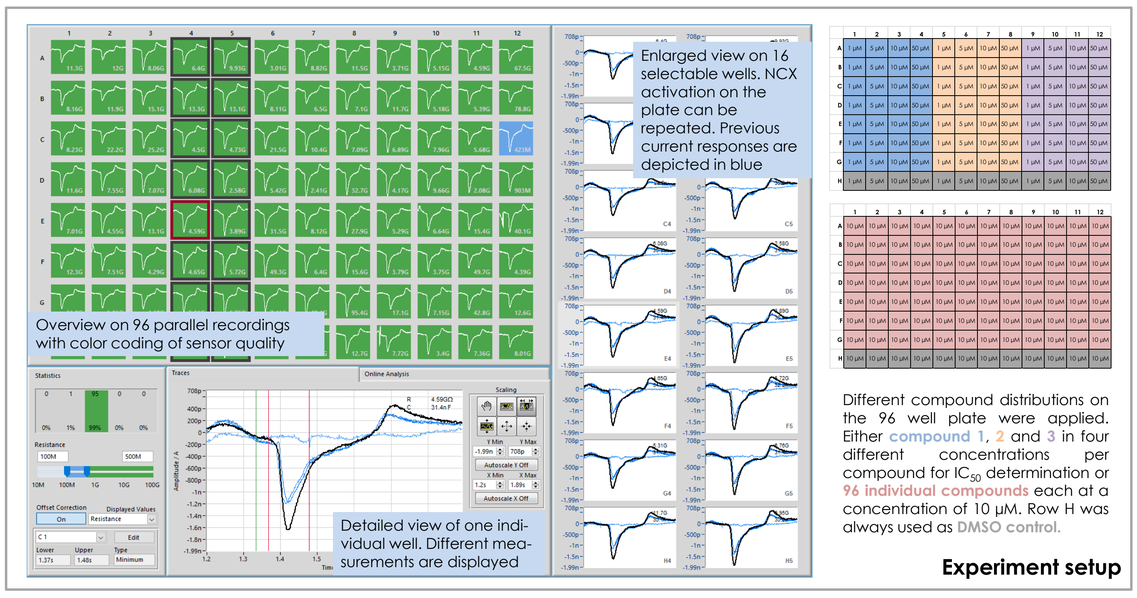
Ncardia Stem cell experts

Cellular Dynamics international a FUJIFILM company

After a rinse with a sodium containing buffer cardiac NCX was activated with 30 µM calcium **(A)**. A transient negative current indicates a net outward current **(B)**. Calcium affinity of NCX recorded from HEK cells (74 µM) was comparable with iCell Cardiomyocytes^2 (125 µM) **(C)**. NCX in HEK cells, iCell Cardiomyocytes^2 and Cor.4U cells was blocked by KB-R7943 in a concentration-dependent manner (**D**) with similar IC_{50} values. The IC_{50} values agreed well with the literature (Matsuda et al, 2001). SEA0400 is a specific blocker of NCX (Matsuda et al, 2001). SEA0400 blocked NCX activated by 30 µM Ca^{2+} with IC_{50} in good agreement with the literature (Matsuda et al, 2001) (**E**).

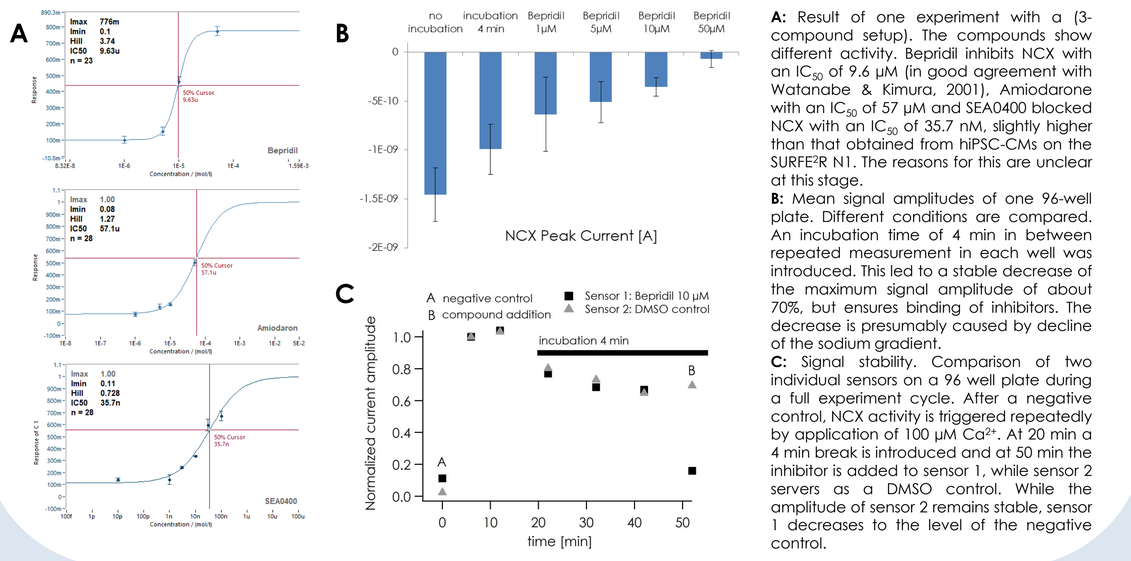
Development of a screening tool for NCX

To screen compounds for an effect on NCX for safety purposes or during development of novel inhibitors a robust method with a high throughput is required. We employed the 96-well based platform SURFE^{2}R 96SE to record NCX activity from stably transfected HEK293 cells. The cells were cultured, harvested and a membrane preparation was performed. This has the advantage that the resulting signal amplitudes are higher, no running cell culture is required and it yields a very high reproducibility. The membrane preparation is defrosted and added to a 96-well sensor where it is recorded by fluidic activation with calcium.



Different compound distributions on the 96 well plate were applied. Either **compound 1**, **2** and **3** in four different concentrations per compound for IC_{50} determination or **96 individual compounds** each at a concentration of 10 µM. Row H was always used as **DMSO control.**

Experiment setup



A: Result of one experiment with a (3-compound setup). The compounds show different activity. Bepridil inhibits NCX with an IC_{50} of 9.6 µM (in good agreement with Watanabe & Kimura, 2001), Amiodarone with an IC_{50} of 57 µM and SEA0400 blocked NCX with an IC_{50} of 35.7 nM, slightly higher than that obtained from hiPSC-CMs on the SURFE^{2}R N1. The reasons for this are unclear at this stage.
B: Mean signal amplitudes of one 96-well plate. Different conditions are compared. An incubation time of 4 min in between repeated measurement in each well was introduced. This led to a stable decrease of the maximum signal amplitude of about 70%, but ensures binding of inhibitors. The decrease is presumably caused by decline of the sodium gradient.
C: Signal stability. Comparison of two individual sensors on a 96 well plate during a full experiment cycle. After a negative control, NCX activity is triggered repeatedly by application of 100 µM Ca^{2+}. At 20 min a 4 min break is introduced and at 50 min the inhibitor is added to sensor 1, while sensor 2 servers as a DMSO control. While the amplitude of sensor 2 remains stable, sensor 1 decreases to the level of the negative control.