The role of Na^+/Ca^{2+} exchanger in development of cardiac alternans

PhD Thesis

Jozefina Szlovák, MSc

Department of Pharmacology and Pharmacotherapy

University of Szeged

Szeged

Hungary

2021

1. Introduction

1.1 Cardiac action potential

The cardiac AP is a characteristic, time-dependent voltage response of the membrane to an appropriate stimulus and formed by coordinated cooperation of various transmembrane ion channels. Several time-dependent Na^+, Ca^{2+}, and K^+ currents contribute to shaping the action potential and its contour is further modified by the effects of intracellular Ca-handling. This complex crosstalk provides that the AP is able to adapt in a wide range of physiological requirements (e.g.: APD shortening in tachycardia), at the same time, the intracellular Ca-mismanagement often causes abnormal action potential waveform that could lead to life-threating arrhythmias. A typical cellular arrhythmia mechanism that involves both the Ca-handling and the transmembrane ionic currents of the AP is the so-called cardiac alternans.

1.2 Action potential and determining ionic currents

1.2.1 Fast inward sodium current (I_{Na})

In cardiomyocytes, the fast inward sodium current (I_{Na}) activated during the zero phase (phase 0) of the AP. When the membrane potential is -60 mV or more positive, the channels open temporarily for a short time (1-2ms), and sodium ions (Na^+) flow into the cell and depolarizing it. The influx of Na^+ is provided by Nav1.5 channels. The large number of channels on the surface membrane provides large current density ensuring fast AP depolarization and impulse conduction. It is important to note that a small fraction of the channels remain opened during the plateau phase of the action potential (i.e. late Na^+ current) and substantially contribute to maintain the plateau phase.

1.2.2 Transient outward potassium current (I_{to})

The fast inward sodium channel inactivation and the transient outward potassium current activation jointly result the early repolarization (phase 1) of the AP. The channel expression varies between tissues and species. The channels are characterized by rapid activation near to -30 mV and rapid inactivation. Potassium ions (K^+)

migrate into the extracellular space for a short time and it leads to a rapid repolarization

1.2.3 Calcium current (I_{Ca})

Inward Ca^{2+} current has two main types (T and L type) in myocardium. The L-type ($I_{Ca,L}$) channel (Cav1.2) has more significance in the ventricular AP, while the T-type primarily operates in nodal tissue such as sinus-node and atrioventricular node where initializes the phase 0 depolarization. The L-type Ca^{2+} channel function is characterized by rapid activation at -40 mV and a relatively slow inactivation which provides the plateau phase of AP (phase 2). I_{CaL} has crucial importance in the Ca^{2+} cycle, since the Ca^{2+} influx through the channel triggers the ryanodine receptors providing Ca^{2+} release from the sarcoplasmic reticulum (SR) . The operation of the channel is regulated by cAMP, extracellular Ca^{2+} and other factors.

1.2.4 The rapid delayed rectifying potassium current (I_{Kr})

These channels (in human HERG) open during depolarization when the voltage reaches the -20 mV or more positive, and it deactivates relatively slowly which is important in maintaining the plateau phase (phase 3) of the AP. It can be blocked by dofetilide leading to prolongation of the APD. The I_{Kr} is considered as the

most important repolarizing current and the primarily player of the repolarization reserve.

1.2.5 The slow delayed rectifying potassium current (I_{Ks})

The I_{Ks} current is provided by the Kv7.1 channel but the β subunit shows interspecies variation. I_{Ks} is activated slowly at a voltage near to -30 mV or more positive values and the current deactivates quickly. However, I_{Ks} contributes to the repolarization process under normal condition, its crucial importance is showed up in the repolarization reserve.

1.2.6 Inward rectifier potassium current (I_{K1})

When the membrane potential is more negative than -40 mV the voltage-dependent I_{K1} (Kir2.1) channels open and play a crucial role in the terminal phase of repolarization. The current exerts strong inward rectification caused by intracellular Mg^{2+} and polyamines.

1.2.7 Sodium-calcium exchanger (NCX)

The NCX antiporter delivers Na^{+} and Ca^{2+} ions between the intra- and extracellular space depending on the actual intracellular levels of these ions and the membrane

voltage. Two main modes of operation are known: the forward mode transports 1 Ca^{2+} out and 3 Na^{+} in the cell, in contrast, the reverse mode moves Ca^{2+} into the cell and 3 Na^{+} out. These ion movements can be measured as inward and outward currents (I_{NCX}).

1.2.8 Na^{+}/K^{+}-ATP-ase

Na^{+}/K^{+}-pump is involved in the restoration of normal intracellular Na^{+} and K^{+} levels after the AP. As it works against the electrochemical gradient, its operation requires energy. It pumps 3 Na^{+} ions out and 2 K^{+} in generating net outward current that slightly contributes to the repolarization ($I_{Na/Kpump}$).

1.3 Cardiac intracellular Ca^{2+} homeostasis

Cardiac intracellular Ca^{2+} cycling provides stable and flexible Ca^{2+} balance for the cardiac muscle contraction. During the plateau phase of the AP the opening of the L-type Ca^{2+} channels provides Ca^{2+} influx that leads to release of Ca^{2+} from the SR. The Ca^{2+} release (Ca^{2+} transient (CaT)) provides available Ca^{2+} for the contraction machinery. Under physiological condition the Ca^{2+} balance requires that neither Ca^{2+} loss nor Ca^{2+} gain happen. In order to secure this, the Ca^{2+} influx must equal to the Ca^{2+} efflux and the Ca^{2+} release must be equal to the amount of Ca^{2+} sequestered back to the SR.

1.3.1 Ca^{2+} influx

The L-type Ca^{2+} current is activated when the AP reaches -40 mV. Ca^{2+} ions enter into the cell, and bound to the ryanodine receptors (RyR) triggering the Ca^{2+} release from the sarcoplasmic reticulum (SR). This process is called calcium induced calcium release (CICR). The released calcium is proportional with the calcium influx, so the inflowing Ca^{2+} defines the calcium release and in line with this the Ca^{2+} content of the SR. Inactivation of the Ca^{2+} current occurs in two ways: the repolarization inactivates the channels in voltage dependent manner (voltage-dependent inactivation), and the other way is the calcium dependent inactivation (CDI) which is regulated by the released Ca^{2+} indirectly, through negative feedback mechanism via calmodulin (CaM).

1.3.2 Ca^{2+} release

Ryanodine receptors are located on the surface of the SR which. RyR has 3 isoforms, in myocardium RyR type 2 (RyR2) are expressed. The receptor has an N-terminal region, containing regulative domains to control opening and closing by phosphorylation spaces. The closed state of the receptor is stabilized by calstabin and it also controls the opening period. During systole the RyR2 channels activated by the Ca-influx, thus the intracellular Ca^{2+} rapidly increases and activates adjacent are ryanodine channels, that results in a rapid rise of the $Ca^{2+}{}_{i}$

from the resting 100 nmol/l concentration to the 1 μmol/l maximum.

1.3.3 Ca^{2+} reuptake and relaxation

In well-functioning myocardial cells, the proper relaxation requires fast dissociation of the Ca^{2+} from the TnC. During relaxation, sequestration of the intracellular Ca^{2+} occurs mainly through active Ca^{2+} uptake by the SR, through the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a). SERCA function is regulated by phospholamban protein (PLN) which is activated by phosphorylation, leading to a decreased interaction between the PLN-SERCA2a and improved transport rate. Ca^{2+}, adenosine-triphosphate (ATP), Mg^{2+}, and optimal pH are required for normal SERCA2a function. About 70% of the released Ca^{2+} is taken back to the SR. The remaining 30% is removed by the forward mode of the NCX.

1.4 Cardiac alternans

Cardiac alternans develop at rapid heart rate showing beat-to-beat oscillation of the action potential duration, and calcium transient amplitude as well. At the same time typical T-wave alternans (TWAs) appeared on the ECG with microvolt deviations. Alternans change the cardiac tissue repolarization pattern by influencing the AP duration and diastolic interval, therefore transform

the physiological repolarization providing enhanced APD heterogeneity. In the 1990s, clinical studies have been connected the TWAs with increased risk for arrhythmias. However, the development of cardiac alternans is not fully clarified. The exact mechanism is still disputed but APD restitution and calcium handling have significant role in the process.

1.4.1 The role of cardiac alternans in arrhythmogenesis

Cardiac alternans have two important types at rapid heart rate. In spatially *concordant* case the AP and calcium transient alternate in the same phase in all regions of the heart. In that case, for a given beat the AP duration and calcium transient amplitude both large/long and small/short in the tissue. Since the effective refractory period alternates in synchronized way, the dispersion of refractoriness and repolarization is only slightly changed. Therefore, spatially concordant alternans less predispose to arrhythmias. In contrast, *discordant* alternans has more importance in arrhythmogenesis. Concordant alternans can be transformed into discordant by the rising heart rate, premature beats or damaged tissue which provides a physical barrier in conduction. During discordant alternans the action potential duration and calcium transient amplitude are out-of-phase in the neighboring

tissues therefore it increases APD heterogeneity and predispose arrhythmias.

1.4.2 Cellular mechanism of cardiac alternans

There are two hypotheses regarding the underling mechanism of alternans. In 1968 Nolasco and Dhalen hypothesized that the development of APD alternans is governed by APD restitution. APD restitution measures the changes of APD in the function of the previous diastolic interval (DI). The steepness of restitution curve is influenced by the recovery of all ion channels during the action potential. The recovery kinetics of the ion channels may affect APD restitution via diastolic interval. Nolasco and Dhalen observed that for a given cycle length (CL) APD alternans appear when the APD restitution curve is larger than 1.

The second hypothesis claims that the calcium handling mismanagement can also induce APD alternans since the membrane potential and intracellular Ca^{2+} are coupled. This theory is the so called Ca^{2+}-driven hypothesis. Alternating Ca^{2+} transients are able to cause APD alternans via enhancing the inward NCX current while it reduces the I_{CaL} due to Ca^{2+}-dependent inactivation.

2. Aims of the study

- Effect of selective NCX inhibition on APD and CaT alternatives, investigation of the role of restitution
- Effect of ORM-10962 on sinus node frequency and intracellular Ca^{2+} level
- Effect of carbachol on activated ATP-dependent K^{+} current

3. Results

3.1 APD and CaT alternans are attenuated via ORM-10962

In canine ventricular papillary muscle APD alternans were measured at two repolarization levels: APD_{25} and APD_{80}. Under control conditions APD alternans are clearly visible at rapid frequency. Application of 1μM ORM-10962 significantly attenuated APD_{25} alternans at all basic cycle length. APD_{80} level alternans were also decreased at basic cycle length of 250 ms and 230 ms.

In order to supplement the electrophysiological recordings of alternans, calcium movements were additionally measured in isolated cells. It was found that transient amplitude alternans were potently and significantly attenuated after selective NCX inhibition by using ORM-10962 at BCL of 230 ms with a trend towards significance at 250 ms bcl.

3.2 Alternans attenuation via ORM-10962 is independent from APD restitution

In the next experimental set, we investigated the possible role of APD restitution in alternans development. APD restitution was measured by using the classic S1-S2 protocol. Under control condition, all hearts exerted restitution slope larger than >1 and there was a non-significant trend towards restitution flattening with ORM-10962.

3.3 ORM-10962 extends postrepolarization refractoriness

In the results of S1-S2 protocol, ORM-10962 extended the duration of postrepolarization refractoriness (PRR), i.e., the interval following APD_{80} where the tissue is still refractory.

3.4 Na^+/Ca^{2+}exchanger inhibition reduces spontaneous sinus-node automaticity and increases Ca^{2+}i level

Literature data and our experiments demonstrate that cardiac alternans are in intimate relationship with pacing frequency. Since it is well-known that the inward NCX current has a crucial role in the diastolic depolarization of the sinus-node cells one can speculate that a possible reduction in the sinus frequency may indirectly facilitate the "anti-alternans" effect of selective NCX inhibition. This possibility was investigated in spontaneously beating isolated sinus-node cells from rabbit right atrium.

In isolated SAN cells the spontaneous frequency of the sinus node cells decreased (CL: 455.6 ± 32 ms *vs.* 493.0 ± 38 ms; Δ = 8.1 ± 1.8% $p < 0.05$) while the diastolic Ca^{2+} level increased after ORM-10962 treatment (70±11 nM *vs.* 130±24 nM; $p<0.05$, n = 10 ;). Furthermore, considerable increase in the transient amplitude (312 ± 37 nM *vs.* 568 ± 85 nM; $p < 0.05$, n = 10) was observed, which was nearly doubled (82.1 ± 22%) in response to ORM-10962 application compared to the control value.

3.3 Carbachol decreased the pinacidil-induced current activation

Beyond the Ca^{2+}-dependent currents (such as I_{CaL}, I_{NCX}), the role of other currents in the APD alternans development cannot be ruled out. It is known that glycolytic inhibition or myocardial infarction promotes the development of cardiac alternans. Under this condition the normal ATP formation is decreased due to tissue hypoxia, therefore the activation of the ATP-dependent K-channels could markedly contribute to the action potential shortening and repolarization heterogeneity.

In our ionic current measurements, voltage ramps were used from a holding potential of -90 mV. Membrane potential was hyperpolarized to -120 mV, and then was slowly (over 36 s) depolarized to 60 mV. Ionic currents were analyzed and compared at 0 and +30 mV [63]. Carbachol did not change the control current when it was applied without pinacidil (0 mV - control: 0.20±0.2 pA/pF vs 3 μM carbachol: 0.32±0.2 pA/pF, n=6 and +30 mV - control: 0.55±0.4 pA/pF vs 3 μM carbachol: 0.74±0.3 pA/pF, n=6). In contrast, when 5 μM pinacidil was applied first, subsequently employed carbachol significantly reduced the current at both voltages. (0 mV

– control: 0.24±0.2 pA/pF 5 μM pinacidil: 2.03±0.3 pA/pF 3 μM carbachol: 1.51±0.4 pA/pF, n=8, $p < 0.05$).

4. Discussion

Main findings of the thesis:

1) Selective NCX inhibition by 1 μM ORM-10962 attenuates AP alternans primarily at APD_{25} levels.

2) Selective NCX inhibition suppresses the alternans of CaT amplitude

3) Selective NCX inhibition extends postrepolarization refractoriness but do not influence the restitution steepness

4) Selective NCX inhibition increases the intracellular Ca^{2+} level in spontaneously beating sinus node cells

5) The muscarinerg antagonist carbachol inhibits the activated IK_{ATP} current.

4.1 Selective NCX blockade attenuates cardiac alternans

Cardiac alternans have been shown to have significant role in the onset of ventricular fibrillation. The

underlying mechanism, however, is not fully clarified so far. The first proposed mechanism was based on the restitution steepness. The authors claimed that alternans will develop when the APD restitution steepness is larger than 1. This theory was termed as voltage-driven mechanism. Later, the voltage-driven mechanism was criticized by several laboratories, and the underlying mechanism was suggested to be rather Ca^{2+}-driven.

The NCX has crucial importance in the normal Ca^{2+}-cycling, due to extrusion of intracellular Ca^{2+} equal to Ca influx. During Ca-efflux a net inward current is generating, therefore the NCX represents a link between intracellular Ca^{2+}-movements and membrane voltage. Considering this dual nature of NCX, it was suggested that NCX may have an important role in the alternans development.

4.2 Na^{+}/Ca^{2+} exchanger inhibition markedly increases the Ca^{2+}_{I} level

The sinus-node pacemaking has one of the most interesting history in the cardiac electrophysiology. The exact mechanism of the spontaneous automaticity was matter of debate for 25 years. Initial concepts claimed that activation of the principal K-current I_{Kr} provides the repolarization and maximal diastolic depolarization and its deactivation provides possibility to the slow depolarization achieved by slow inward leakage currents.

This theory was replaced after the discovery of the pacemaker current (funny-current, I_f). The concept announced that the I_f is the primary drive of the spontaneous diastolic depolarization, as having a hyperpolarization-activated kinetics, and shows strong cAMP-dependence to respond to adrenergic stimulation. The I_f-driven pacemaking had dominant role in sinus-node automaticity until the late 90's when the importance of the intracellular Ca^{2+} handling was supported by an increasing number of publications. These reports focused to the role of inward (forward) NCX as an essential player transforming Ca^{2+}-changes to membrane potential depolarization. A break-through discovery was published in 2004 when spontaneous, rhythmic, local-Ca^{2+} releases were found preceding the "normal" Ca^{2+} release from the SR. These local events (LCR) generate NCX current to contribute to the diastolic depolarization. Since the rhythmicity of the LCRs may provide rhythmic sinus-node pacemaking, it was termed 'Ca-clock'.

At the same time, it was indisputable that selective inhibition of various transmembrane ionic currents (I_f, I_{Kr}, I_{CaL}, I_{CaT}) substantially influence pacemaking. Several publications were released demonstrating coupling between surface membrane ion channels and Ca^{2+} handling, leading to the current, widely accepted theory: 'coupled-clock' mechanism. The coupled-clock concept claims that that surface membrane ion channels as well as intracellular Ca^{2+}-handling work together,

neither is dominant, but provides a redundant, fail-safe, complex mechanism.

In our experiments, we found a slight but statistically significant (~8%) decrease in the cycle length of sinus node cells. This effect, however lower than was expected, it was coupled with an increase of the diastolic Ca^{2+} and the transient amplitude. The selective NCX inhibition caused similar diastolic Ca^{2+} changes compared to the Yaniv model predicted, however, in contrast with modeling, we found markedly increased Ca^{2+} transient amplitude which is generally expected after decreased rate of Ca^{2+} extrusion. The observed quantitative discrepancy between experiments and modeling may indicate that the extent of NCX inhibition in the experiments could be larger than 41%.

We can speculate that the increasing intracellular Ca^{2+} is known to facilitate the inactivation of the L-type Ca^{2+} current as a part of the autoregulation. The gain of the $[Ca^{2+}]_i$ may indirectly shortens the CL which means two parallel, counteracting effect of selective NCX inhibition: the inhibition of the inward NCX current may reduce the actual frequency by suppressing its contribution to the DD, however it is partially compensated for the CL abbreviating effect of increased $[Ca^{2+}]_i$. Furthermore, the I_f may also contribute in the

limitation of the ORM effect: i) a theoretical possibility exists that ORM-induced Ca^{2+} elevation may increase the I_f, however this was ruled out by a previous work (ii) It was reported that SAN myocytes express Ca^{2+}-activated adenylate cyclase isoform, which might raise cAMP (and I_f) in response to NCX blockade.

Taking together these results, it is feasible that selective NCX inhibition may cause several concomitant and counterintuitive changes in the sinus node cells, because NCX inhibition not only inhibits the NCX current during the DD, but modulates the Ca^{2+} levels of the cells. Ultimately, it results marginal decrease in sinus-node frequency, but it may have potential significance in the alternans-attenuation effect of selective NCX inhibition. In our AP alternans experiments we have never observed APD alternans at BCL of 300 ms, in contrast, alternans were always found at 250 ms. This may indicate a very narrow frequency border between normal and alternating AP behavior. In this case, a mild bradycardic effect may have important role in attenuation of alternans.

4.3 Acetylcholine inhibits the IK-ATP in canine ventricular myocytes

It was reported that alternans are often developed in ischemia-reperfusion injury. This finding raises the

possibility that $I_{K\text{-}ATP}$ current that exerts a well-known activation in ischemia, may contribute to the alternans mechanism, at least via increasing the APD heterogeneity. In contrast, the direct role of the $I_{K\text{-}ATP}$ in the development of alternans is controversial. It was reported that $I_{K\text{-}ATP}$ expression exerts down-regulation during myocardial infarction. Nicorandil (an $I_{K\text{-}ATP}$ opener) restored the APD and flattened the restitution curve and suppressed the spatially discordant arrhythmias.

In line with this, it was showed by early studies, that increased parasympathetic tone is able to reduce ischemia-related arrhythmias. In our experiments, as was expected, we found significant increase of $I_{K\text{-}ATP}$ after application of pinacidil (almost 10-fold increase). It was also demonstrated that this large increase of the current abbreviates the ventricular action potential. Interestingly, our novel finding is that muscarinergic-agonist carbachol is able to reduce the $I_{K\text{-}ATP}$ current. The underlying mechanism could be related to the change of intracellular signaling pathways such as PKC however, it requires further experiments. These interesting results may provide a further aspect of antiarrhythmic effect of increased parasympathetic tone in ischemia. This effect of parasympathetic tone could be achieved, at least partially, via attenuation the $I_{K\text{-}ATP}$-mediated increased alternans propensity.

PUBLICATIONS RELATED TO THE THESIS

I. **Szlovák J**, Tomek J, Zhou X, Tóth N, Veress R, Horváth B, Szentandrássy N, Levijoki J, Papp JG, Herring N, Varró A, Eisner DA, Rodriguez B, Nagy N.

Blockade of sodium-calcium exchanger via ORM-10962 attenuates cardiac alternans.
J Mol Cell Cardiol. 2020 Dec 28; 153:111-122.

II. Kohajda Z, Tóth N, **Szlovák J**, Loewe A, Bitay G, Gazdag P, Prorok J, Jost N, Levijoki J,Pollesello P, Papp JG, Varró A, Nagy N.

Novel Na^{+}/Ca^{2+} Exchanger Inhibitor ORM-10962 Supports Coupled Function of Funny-Current and Na^{+}/Ca^{2+} Exchanger in Pacemaking of Rabbit Sinus Node Tissue
Front Pharmacol. 2020 Jan 29; 10:1632.

III. Magyar T, Árpádffy-Lovas T, Pászti B, Tóth N, **Szlovák J**, Gazdag P, Kohajda Z, Gyökeres A, Györe B, Gurabi Z, Jost N, Virág L, Papp JG, Nagy N, Koncz I.

Muscarinic agonists inhibit the ATP-dependent potassium current and suppress the ventricle-Purkinje action potential dispersion
Can J Physiol Pharmacol. 2020 Nov 26; 1-7.

Further publications

I. Pászti B, Prorok J, Magyar T, Árpádffy-Lovas T, Györe B, Topál L, Gazdag P, **Szlovák J**, Naveed M, Jost N, Nagy N, Varró A, Virág L, Koncz I.

Cardiac electrophysiological effects of ibuprofen in dog and rabbit ventricular preparations: possible implication to enhanced proarrhythmic risk
Can J Physiol Pharmacol 2021 Jan;99(1):102-109

II. Orvos P, Kohajda Z, **Szlovák J**, Gazdag P, Árpádffy-Lovas T, Tóth D, Geramipour A, Tálosi L, Jost N, Varró A, Virág L.

Evaluation of Possible Proarrhythmic Potency: Comparison of the Effect of Dofetilide, Cisapride, Sotalol, Terfenadine, and Verapamil on hERG and Native IKr Currents and on Cardiac Action Potential
Toxicol Sci.2019 Apr 1; 168 (2):365-380.

III. Gazdag P, Oravecz K, Acsai K, Demeter-Haludka V, Ördög B, **Szlovák J**, Kohajda Z, Polyák A, Barta BA, Oláh A, Radovits T, Merkely B, Papp JG, Baczkó I, Varró A, Nagy N, Prorok J.

Increased Ca^{2+} content of the sarcoplasmic reticulum provides arrhythmogenic trigger source in swimming-induced rat athlete's heart model.
Sci. Rep.2020 Nov11; 10 (1):19596.

Acknowledgement

I would like to express my sincere thanks to my PhD supervisor **Dr. Norbert Nagy**, for his professional advice, continuous support of my work and for introducing me to the research field of cardiac cellular electrophysiology.
I appreciate very highly the advice, comments and support of **Professor Julius Gy. Papp** MD, DSc, academician.
I am also grateful to **Professor András Varró** MD, DSc and **Dr. István Baczkó** for providing me the opportunity for conducting research as PhD student at the Department of Pharmacology and Pharmacotherapy, University of Szeged.

I would like to thank to my former supervisior, **Dr. Balázs Ördög** for his personal advices, encouragement.

I would like to thank **Dr. Zsófia Nagy** for her work and personal guidance. I also thank **Dr. Noémi Tóth** and **Péter Gazdag MSc**, **Gergő Bitay, Dr. László Virág, Dr. Norbert Jost**, **Teodóra Hartai MSc**, **Szilvia Déri MSc**, **Dr. Bence Pászti**, **Dr. Tibor Magyar**, **Dr. Tamás Árpádffy-Lovas** for their work and for their support during my PhD studies.

Special thanks to **Zsuzsanna Molnár, Erika Bakó, Anikó Kőrös, Rea Fritz** and **Gábor Girst** for their technical and administrative work.

Finally, I would like to thank and recommend this thesis to my family, friends, for their support and encouragement.