Ca^{2+} dependent regulation of sinoatrial node pacemaking

Ph.D. thesis

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LIST OF PUBLICATIONS

The publications related to the subject of the Ph.D. thesis:

I.) Gergő Bitay, **Noémi Tóth**, Szilvia Déri, Jozefina Szlovák, Zsófia Kohajda, András Varró, Norbert Nagy. The Inhibition of the Small-Conductance Ca^{2+}-Activated Potassium Channels Decreases the Sinus Node Pacemaking during Beta-Adrenergic Activation. *Pharmaceuticals* 2022, https://doi.org/10.3390/ph15030313, (**IF**: 5.863, **Q1**)

II.) **Noémi Tóth**, Jozefina Szlovák, Zsófia Kohajda, Gergő Bitay, Roland Veress, Balázs Horváth, Julius Gy. Papp András Varró, Norbert Nagy. The development of L-type Ca^{2+} current mediated alternans does not depend on the restitution slope in canine ventricular myocardium. *Scientific Reports* 2021, DOI: 10.1038/s41598-021-95299-7 , (**IF**: 4.379, **D1**)

III.) Zsófia Kohajda*, **Noémi Tóth***, Jozefina Szlovák, Axel Loewe, Gergő Bitay, Péter Gazdag, János Prorok, Norbert Jost, Jouko Levijoki, Piero Pollesello, Julius Gy. Papp, András Varró, Norbert Nagy. 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. *Frontiers in Pharmacology 2020,* DOI: 10.3389/fphar.2019.01632 , (**IF**: 5.811, **Q1**)

+1.) **Noémi Tóth**, Axel Loewe, Jozefina Szlovák, Zsófia Kohajda, Gergő Bitay, Jouko Levijoki, Julius Gy. Papp, András Varró, Norbert Nagy. The reverse mode of the Na^{+}/Ca^{2+} exchanger contributes to the pacemaker mechanism in rabbit sinus node cells. *Scientific Reports, under major revision*

Related published abstracts of the "*The reverse mode of the Na^{+}/Ca^{2+} exchanger contributes to the pacemaker mechanism in rabbit sinus node cells*" study:

- Tóth, N; Loewe, A; Kohajda, Zs; Bitay, G; Levijoki, J; Papp, JGy; Varró, A; Nagy, N. Investigation of the reverse Na^{+}/Ca^{2+} exchanger function in the spontaneous automaticity of rabbit sinus node cells. SCRIPTA MEDICA 52 : Suppl.1 p. S16 (2021)
- Tóth, N; Szlovák, J; Loewe, A; Gazdag, P; Bitay, G; Levijoki, J; Papp, Gy; Varró, A; Nagy, N. A reverz Na^{+}/Ca^{2+} kicserélő szerepének vizsgálata a szinusz-csomó spontán automáciájában = The role of reverse Na^{+}/Ca^{2+} exchanger in the sinus node spontaneous automaticity. CARDIOLOGIA HUNGARICA 50 : Suppl. D pp. 168-168. , 1 p. (2020)

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Other publications published under Ph.D. scholarship:

IV.) Zsófia Kohajda, László Virág, Tibor Hornyik, Zoltán Husti, Antia Sztojkov-Ivanov, Norbert Nagy, András Horváth, Richárd Varga, János Prorok, Jozefina Szlovák, **Noémi Tóth**, Péter Gazdag, Leila Topal, Muhammad Naveed, Tamás Árpádffy-Lovas, Bence Pászti, Tibor Magyar, István Koncz, Szilvia Déri, Vivien Demeter-Haludka, Zoltán Aigner, Balázs Ördög, Márta Patfalusi, László Tálosi, László Tiszlavicz, Imre Földesi, Norbert Jost, István Baczkó, András Varró. In vivo and cellular antiarrhythmic and cardiac electrophysiological effects of desethylamiodarone in dog cardiac preparations. *British Journal of Pharmacology* 2022, DOI: 10.1111/bph.15812 , (**IF**: 8.739, **D1**)

V.) István Koncz, Arie O. Verkerk, Michele Nicastro, Ronald Wilders, Tamás Árpádffy-Lovas, Tibor Magyar, **Noémi Tóth**, Norbert Nagy, Micah Madrid, Zexu Lin, Igor R. Efimov.
Acetylcholine Reduces I_{Kr} and Prolongs Action Potentials in Human Ventricular Cardiomyocytes. *Biomedicines* 2022, DOI: 10.3390/biomedicines10020244 , (**IF**: 6.081, **Q1**)

VI.) Tibor Magyar, Tamás Árpádffy-Lovas, Bence Pászti, **Noémi Tóth**, Jozefina Szlovák, Péter Gazdag, Zsófia Kohajda, András Gyökeres, Balázs Györe, Zsolt Gurabi, Norbert Jost, László Virág, Juliu Gy. Papp, Norbert Nagy, István Koncz. Muscarinic agonists inhibit the ATP-dependent potassium current and suppress the ventricle-Purkinje action potential dispersion. *Canadian Journal of Physiology and Pharmacol*ogy 2021, DOI: 10.1139/cjpp-2020-0408 , **(IF:** 2.273**, Q3)**

VII.) Jozefina Szlovák*, Jakub Tomek*, Xin Zhou, **Noémi Tóth**, Roland Veress, Balázs Horváth, Norbert Szentandrássy, Jouko Levijoki, Julius Gy. Papp, Neil Herring, András Varró, David A. Eisner, Blanca Rodriguez, Norbert Nagy. Blockade of sodium-calcium exchanger via ORM-10962 attenuates cardiac alternans. *Journal of Molecular and. Cellular Cardiol*ogy 2021, DOI: 10.1016/j.yjmcc.2020.12.015 , (**IF**: 5.000, **Q1**)

VIII.) **Noémi Tóth**, Alexandra Soós, Alex Váradi, Péter Hegyi, Benedek Tinusz, Anna Vágvölgyi, Andrea Orosz, Margit Solymár, Alexandra Polyák, András Varró, Attila S. Farkas, Norbert Nagy. Effect of ivabradine in heart failure: a meta-analysis of heart failure patients with reduced versus preserved ejection fraction. *Canadian Journal of Physiology and Pharmacol*ogy 2021, DOI: 10.1139/cjpp-2020-0700 , (**IF**: 2.273, **Q3**)

IX.) Zsófia Kohajda, Axel Loewe, **Noémi Tóth**, András Varró, Norbert Nagy. The Cardiac Pacemaker Story-Fundamental Role of the Na^+/Ca^{2+} Exchanger in Spontaneous Automaticity. *Frontiers in Pharmacology 2020,* DOI: 10.3389/fphar.2020.00516 , (**IF**: 5.811, **Q1**)

X.) Axel Loewe, Yannick Lutz, Deborah Nairn, Alan Fabbri, Norbert Nagy, **Noemi Toth**, Xiaoling Ye, Doris H. Fuertinger, Simonetta Genovesi, Peter Kotanko, Jochen G. Raimann, Stefano Severi. Hypocalcemia-Induced Slowing of Human Sinus Node Pacemaking.
Biophysical Journal 2019, DOI: 10.1016/j.bpj.2019.07.037 , (**IF**: 3.854, **D1**)

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1. INTRODUCTION

Normal heart rhythm depends on a precise and regular activity of the sinoatrial node (SAN) that is located in the right atrium of the heart. SAN serves as the primary center of the pacemaker system by initiating the heart beats. Pacemaker cells that create the SAN possesses the characteristic nature of automaticity, since they are able to ignite action potentials (APs) in the absence of external stimuli. Spontaneous AP generation needs a unique cooperation of membrane ion channels and intracellular Ca^{2+} handling. One of the most important question in the cardiac electrophysiology field is 'what is the main iniciator mechanism that ignites the AP of the SAN cells to enable the spontaneous pacemaking of the heart'. Several theories was born to explain the mechanisms underlying the spontaneous rhythm generation, however the earliest ones were not able to fully support the phenomenon.

The APs of SAN cells and AVN cells are different from the APs of the cells in the working myocardium. Phase 0 depolarization is mediated by the openings of the T-type- then the L-type Ca^{2+} channels. The activation of L-type Ca^{2+} channels is slower than the activation of Na^{+} channels in the ventricles, therefore the depolarization slope is less steep in pacemaker cells. Due to the lack of I_{to} activation, phase 1 repolarization is not present in SAN cells. Upon repolarization (phase 3) only the rapid component of the delayed rectifier potassium current (I_{Kr}) is activated and hyperpolarizes the membrane to reach the maximal diastolic potential (MDP). The SAN AP shows a characteristic phase 4 depolarization, where the membrane potential onset is more depolarized (MDP is around -50 – -60 mV) and undergoes a slow diastolic depolarization (DD). DD ends at the take off potential, which is around -40 mV.

The question indicating what is the basic mechanism of SAN pacemaking is still a matter of debate after more than 40 years of intensive research. There is no doubt in the literature that the spontaneous activity of the pacemaker cells is controlled by the DD. However, our knowledge on the exact mechanism underlying this phenomenon is still incomplete, despite the intensive research work of different acknowledged laboratories. The earliest theory of the pacemaking is originated from the 1950s, when Weidmann et al. attributed an important role to the decay of a K^{+} current during the DD in Purkinje fibers. Two types of K^{+}-currents were distinguished: the time-independent, fast repolarizing current I_{K1}, and the time-dependent, slow current I_{K2} which is sensitive to adrenaline. The proposed mechanism of pacemaking was the slow decay of the K^{+}-currents together with slow inward depolarizing currents. When K^{+}-currents are decayed, the inward background currents exceed the repolarizing currents and the membrane is slowly depolarized. In 1979 Dario DiFrancesco discovered the so-called "funny current" (I_f) on Purkinje fibers. "Funny current" got the name from its unconventional

behaviour: the current carries depolarizing current (mix of Na^+ and K^+ ions) but it is activated upon hyperpolarization. Additionally, it was found to be sensitive to adrenaline. It was observed that I_f is able to cause spontaneous depolarizations in Purkinje fibers. An obvious question was emerged: are there two completely distinct mechanisms for the same function between two regions (SAN *vs.* Purkinje fibers) of the heart? A milestone of the SAN pacemaking research was when the I_{K2} was "reinterpreted" and it was demonstrated that I_{K2} is actually the same current as I_f. From 1982 to the beginning of the 2000's the "funny-concept" was the leading mechanism to explain SAN automaticity: hyperpolarization during repolarization activates I_f which depolarizes the membrane during the DD. Additionally, I_f shows dual activation by voltage and cAMP, therefore it also transmits the changes of the autonomic nervous system. With the discovery of I_f the spontaneous rhythm generation seemed to be explained for decades, however later the hypothesis was repeatedly challenged. Noma and Morad showed that complete block of I_f by caesium did not stop the spontaneous activity emphasizing other possible mechanisms than I_f. In 1996 a new possible player in the SAN automaticity was suggested. Rigg and Terrar demonstrated that the inhibition of sarcoplasmic Ca^{2+} releases or refilling decreases the SAN frequency highlighting an important role of Ca^{2+} handling in pacemaking. Lakatta and his collegues identified spontaneous, subsarcolemmal local Ca^{2+} releases (LCR) that appears during the late DD. It was claimed that LCRs produce an inward current by activating the forward Na^+/Ca^{2+} exchanger (NCX) and contribute to the ignition of SAN AP. This concept indicated that the primary mechanism of DD is the NCX mediated Na^+ influx and almost completely ignored the role I_f in SAN pacemaking. Later, this hypothesis was named as the *Ca^{2+} clock theory*, since the sarcoplasmic reticulum (SR) serves as the Ca^{2+} clock itself by generating rhythmic LCRs. From that point an intensive debate emerged between the Ca^{2+} clock hypothesis and the „funny current" hypothesis. The „funny current hypothesis" was later extended to *membrane clock (M-clock) hypothesis* indicating that surface membrane ion channels have pivotal role in pacamaking. Intensive research in this field revealed that intracellular Ca^{2+} and surface membrane ion channels are tightly coupled during pacemaking. This synergistic cooperation forms the latest concept of the so-called *coupled clock hypothesis*. Since SAN pacemaking is based on mutual coupling between intracellular Ca^{2+} handling and membrane potential, Ca^{2+} dependent ionic currents may have pivotal role in establishing connections between these systems.

2. AIMS

Since the mechanism of SAN pacemaking is not fully clarified, the aims of the present study were the followings:

1.) to investigate the function of the forward NCX current by its direct pharmacological inhibition using the novel, selective inhibitor ORM-10962 and analyse the suggested crosstalk between NCX current and I_f in multicellular level of SAN pacemaking,

2.) to investigate the potential existence and functional role of the reverse NCX current in the SAN pacemaker mechanism,

3.) to investigate the role of $I_{K(Ca)}$ in the SAN pacemaking,

4.) to study the arrhythmogenic consequences of enhanced SAN pacemaking on the function of ventricles (i.e. test the possible role of Ca^{2+} handling and restitution in the development of cardiac alternans).

3. RESULTS

3.1 Investigation of forward NCX function and the NCX-I_f coupling in SAN pacemaking

The role of NCX have been suspected in the coupled clock concept of SAN pacemaking since the NCX mediated inward current is directly translated to membrane potential changes via the operation of forward mode of the exchanger. However, direct pharmacological experiments have not been performed so far because of the lack of selective inhibitor. In the first part of this study, the role of forward NCX and the potential strong cooperation of I_f-NCX were tested with the selective NCX blocker, ORM-10962.

3.1.1 NCX inhibition exerted CL prolongation on SAN tissue

Spontaneous automaticity (i.e. spontaneous APs) on SAN tissue was measured by conventional microelectrode technique. 1 μM selective NCX inhibitor ORM slightly, but statistically significantly increased the cycle lengths (CL) (455.6 ± 32 ms vs. 493.0 ± 38 ms; Δ = 8.1 ± 1.8% $p < 0.05$, n = 16/16 hearts) without any influence on the action potential duration (APD) (94.3 ± 6.7 ms vs. 96.7 ± 5.9 ms) or the CL variability (7.6 ± 1.2 ms vs. 8.1 ± 1.3 ms). The slope of the DD phase was significantly reduced after ORM application (15.7 ± 3.1 mV/s vs. 10.9 ± 2.8 mV/s; n = 14/14; $p < 0.05$).

3.1.2 NCX inhibition exerted larger CL lengthening effect when I_f was previously impaired

The supposed coupling between I_f and NCX was tested by the subsequent application of the I_f inhibitor ivabradine (IVA) and NCX blocking ORM. The effect of NCX inhibition was significantly larger when I_f was previously inhibited compared with the condition when ivabradine was not applied (8.1 ± 1.88% *versus* 17.1 ± 2.5%; $p < 0.05$, ANOVA, Bonferroni *post hoc* test). A clear, gradual increasing ORM effect was observed on the CL with combined increasing concentration of ivabradine (1 μM ORM effect in the presence of 0 μM IVA: 8.1 ± 1.88%; in the presence of 0.5 μM IVA: 9.6 ± 2.3%; in the presence of 3 μM IVA: 17.1 ± 2.5%). Ivabradine significantly increased the CL both with the concentration of 0.5 and 3 μM ($p < 0.05$, ANOVA, Bonferroni *post hoc* test).

3.1.3 Repolarization inhibition induced bradycardia does not facilitate the CL lengthening effect of NCX inhibition

We studied how does an other mechanism that does not directly involve the depolarizing currents but also lead to bradycardia would influence the effect of NCX inhibition in SAN. I_{Kr} was blocked by 100 nM dofetilide (DOF) causing significant CL prolongation (control: 489.3 ± 31 ms → 100 nM dofetilide: 649.1 ± 40.2 ms). Subsequent administration of 1 μM ORM resulted in the same effect compared with the individual application of ORM (7.2 ± 1.8% *vs.* 8.1 ± 1.8%). It is very important to highlight that the effect of dofetilide on CL was approximately similar to the effect of 3 μM ivabradine. Dofetilide mediated CL increase was an APD increase induced effect, while ivabradine caused CL prolongation by influencing only the DI with no change in the APD. NCX inhibition and I_f inhibition both influence the DI by decreasing its slope, while dofetilide does not have any effect on the DD slope. This could be the reason why the CL prolongating effect of ORM in combination with prior application of dofetilide was not additive.

3.1.4 Reduction of $[Ca^{2+}]_i$ increases the CL lengthening effect of I_f inhibition

The potential effect of reduction in SR Ca^{2+} release on the effect of ivabradine was also tested. After the control recording, 5 μM ryanodine (RYA) was applied to prevent the Ca^{2+} release induced augmentation of the forward NCX current. Ryanodine exerted a significant CL prolongation (437.8 ± 20.3 ms *vs.* 499.8 ± 10.4 ms; $p < 0.05$, $n = 6/6$). ORM was susequently applied and caused a moderate CL increase. However, when 3 μM ivabradine was added top of the ORM, it markedly and significantly increased the CL of SAN tissue

(520.8 ± 29.9 ms *vs.* 726.6 ± 39.8 ms; $p < 0.05$, n = 6/6). I_f inhibition resulted in larger CL prolongation in the presence of impaired Ca^{2+} handling (ryanodine and ORM application, 42.4 ± 5.7%, $p < 0.05$, Student's T-test) compared to the individual adminsitration of ivabradine.

In other set of experiments, suppressed $[Ca^{2+}]_i$ was achieved by the application of reduced $[Ca^{2+}]_o$ external solution (0.9 mM $CaCl_2$). Reduced extracellular Ca^{2+} prolonged the CL which was further lengthened after the application of 3 μM ivabradine (control: 469 ± 39.5 ms → 0.9 mM $[Ca^{2+}]_o$: 515.8 ± 40.8 ms → 3 μM IVA: 777 ± 58.7 ms; $p < 0.05$, n = 6/6 hearts). Ivabradine has improved CL lengthening effect when extracellular Ca^{2+} is low compared with normal extracellular Ca^{2+} settings (51.1 ± 5.1% *versus* 20.99 ± 4.1%, $p < 0.05$; Student's t-test).

3.1.5 Subsequent inhibition of NCX and I_f increases the CL variability

Stability and rhythmicity of SAN pacemaking is essential for physiological cardiovascular function. CL variability was assessed by analysing 30 consecutive spontaneous APs. Application of 1 μM ORM and 3 μM ivabradine individually decreased the spontaneous frequency without considerably changing the CL variability. The concomitant application of 5 μM ryanodine and5 μM rynaodine + 1 μM ORM showed an enhancement in the CL varibility, however it was not statistically significant. In contrast, additional adminstration of 3 μM ivabradine remarkably and statistically significantly increased the CL variability.

3.2 Investigation of reverse NCX function in SAN pacemaking

Our experimental results support the coupled clock hypothesis and the essential role of forward NCX in the clock-like oscillatory system. However, it is well known that the direction of the ionic transport through the NCX is reversible and influenced by several factors. As it was mentioned before, in cardiac research regarding the spontaneous automaticity of SAN the focus was entirely on the forward operation of the NCX. To our best knowledge, there is only one computational study that attributed important role to reverse function. Therefore, in the other part of this study we investigated the potential existence and functional role of the reverse NCX current in the SAN pacemaker mechanism.

3.2.1 Experimental validation of 2 mM and 8 mM $[Na^+]_{pip}$ groups in the measurement of reverse NCX current

To study the possible role of reverse NCX current in SAN pacemaking two experimental groups (2 mM and 8 mM $[Na^+]_{pip}$) were established. 8 mM NaCl ensures the approximately

physiological Na^+ level of SAN cells, while in the other group the pipette NaCl concentration was reduced to 2 mM in order to diminish the reverse mode of the NCX, without completely eliminating the exchanger function. Firstly, conventional NCX voltage ramp protocol was used to study the reverse NCX current in 2 mM and 8 mM $[Na^+]_{pip}$ conditions. No outward (i.e. reverse) NCX current was found in the presence of 2 mM $[Na^+]_{pip,}$ while NCX blocker $NiCl_2$ and ORM dissected a notable outward component with 8 mM $[Na^+]_{pip.}$ The effect of the selective NCX inhibitor ORM-10962 was also assessed and compared between the different experimental conditions, and no Na^+ dependent effect of ORM was found.

3.2.2 Characterization of NCX current under the SAN AP

A canonical SAN AP waveform was used as a command potential to investigate the NCX current under the entire AP. In order to fully inhibit the NCX current 10 mM $NiCl_2$ was applied after the control recording. NCX current was gained as a subtracted current from the control current and the current remained after the application of $NiCl_2$. In the presence of 2 mM $[Na^+]_{pip}$ a negligible 0.33±0.3 pC outward current was observed (n=5), while in the presence of 8 mM $[Na^+]_{pip}$ the outward current was significantly larger (2.1±0.3 pC, n=7, $p<0.05$). This value is almost identical with the prediction of the Maltsev-Lakatta model (2.45 pC). This experimental condition allows estimating the total carried charges via the reverse NCX, however lacks the Ca^{2+} release that is fundamental driving force of NCX. Therefore, the same protocol was applied on SAN cells with enabled Ca^{2+} release. Aiming to avoid the I_{CaL} suppressing effect of $NiCl_2$, in these experiments ORM was used to assess the reverse NCX current. Similar to the results with $NiCl_2$, an outward component of the NCX current appeared at the beginning of the AP, and it was absent in the 2 mM $[Na^+]_{pip}$ group.

3.2.3 In the presence of active reverse NCX the Ca^{2+} transient is larger

Diastolic Ca^{2+} level and CaT amplitude were analysed under the command AP. CaT amplitude was found to be significantly higher with functioning reverse NCX (i.e. 8 mM $[Na^+]_{pip}$) than with no reverse NCX function (2 mM $[Na]_{pip}$: 308±37 nM, n=14 *vs* 8 mM $[Na]_{pip}$: 539±52 nM, n=14; $p<0.05$, ANOVA with Tukey *post hoc* test). The diastolic Ca^{2+} level showed no difference comparing the groups (2 mM $[Na^+]_{pip}$: 117±14 nM, *vs* 8 mM $[Na^+]_{pip}$: 149±24 nM, n=14-14; $p<0.08$, ANOVA with Tukey *post hoc* test), while the half-relaxation time was significantly longer when reverse NCX was active (2 mM $[Na^+]_{pip}$: 112±5 ms *vs* 8 mM $[Na^+]_{pip}$: 146±9 ms; n=14-14, $p<0.05$; ANOVA with Tukey *post hoc* test).

3.2.4 Reverse NCX activity enhances the SR Ca^{2+} content

The larger CaT amplitude found with functioning reverse NCX suggests increased SR Ca^{2+} content. Ca^{2+} content of the SR was measured by rapid administration of 10 mM caffeine. Caffeine evoked an inward current and a caffeine induced CaT. The SR Ca^{2+} concentration was calculated from the integral of the caffeine induced inward current with normalization to the cell capacitance. Functioning reverse NCX increased the SR Ca^{2+} content (8 mM $[Na^+]_{pip}$: -3.7±0.5 C/F, n=11; 2 mM $[Na^+]_{pip}$: -2.3±0.3 C/F, n=11; $p<0.05$, unpaired t-test).

3.2.5 Active reverse NCX enhances the spontaneous AP firing rate

Spontaneous frequency of SAN cells were measured using whole cell patch clamp technique with current clamp mode. The frequency was calculated by analysing the CLs of 30 consecutive APs. We found higher frequency, i.e. shorter CLs in the presence of active reverse NCX (8 mM $[Na^+]_{pip:}$ 369±15 ms *vs* 2 mM $[Na^+]_{pip}$ 463±38 ms; $p<0.05$, n=8-8).
Steeper DD (0.12±0.02 mV/ms *vs* 0.07±0.01 mV/ms; $p<0.05$, n=8-8) and shortened APD (189±3 ms *vs* 232±11 ms; $p<0.05$, n=8-8) were found in the presence of 8 mM $[Na^+]_{pip}$. CaT amplitude was larger with 8 mM $[Na^+]_{pip\ (}$420±52 nM *vs* 250±22 nM; $p<0.05$, n=8-8).

3.3 $I_{K(Ca)}$ has no role in the spontaneous SAN pacemaking

Spontaneous AP measurements revealed enhanced firing rate with 8 mM $[Na^+]_{pip}$**,** while the APD also shortened. This interesting APD shortening could be a consequence of the higher $[Ca^{2+}]_i$ induced faster I_{CaL} inactivation, or due to an additional repolarizing current activation via possible activation of the small-conductance Ca^{2+}-activated K^+-channels, as was reported in a previous study[83]. Since $I_{K(Ca)}$ carries a functional repolarizing current that depends on the intracellular Ca^{2+} level, it is possible that $I_{K(Ca)}$ also influence the spontaneous frequency. We measured the possible contribution of $I_{K(Ca)}$ in the pacemaking under the previously defined SAN AP command. Control current was recorded in Tyrode's solution, then 100 nM apamin was added to dissect the apamin sensitive $I_{K(Ca)}$ current. Negligible apamin sensitive current was found in normal conditions. To further test the assumption, spontaneous APs were measured in this case as well. APs were measured by perforated patch clamp technique. Apamin failed to alter any parameter of the spontaneous APs (control → apamin; cycle length: 391 ± 30 ms → 388 ± 33 ms; cycle length variability: 43 ± 10 ms → 41 ± 13 ms; APD: 176 ± 17 ms → 193 ± 25 ms; slope of diastolic depolarization: 0.08 ± 0.01 mV/ms → 0.08 ± 0.01 mV/ms; n = 7). This finding and the negligible current found under the AP support that $I_{K(Ca)}$ has no role in the spontaneous pacemaking.

3.4 Cardiac alternans show a restitution independent nature that is associated with enhanced frequency

APD restitution and AP alternans protocol were measured on intact subendocardial tissue**.** The S1S2 restitution was recorded at both BCL of 1000 and 500 ms. Alternans were observable in all cases (n=20) with clear frequency threshold, i.e. when the pacing length was equal or shorter than 250 ms. Alternans were maintained from the start to the end of the protocol without any decline in the amplitude of APD oscillation. Restitution slopes and corresponding amplitude of APD alternans were compared. Our results show that alternans were inducible even if the restitution slope was smaller than 1, and data show mainly weak and in some cases moderate correlations between the restitution slope and alternans amplitude at different repolarization levels of APD. Parallel with the demonstration of negligible role of restitution slope in the development of cardiac alternans, strong correlation was found between CaT and AP alternans amplitude. Stimulus AP command from a CL of 250 to 210 ms was applied to ventricular cardiomyocytes and CaTs were measured. A close relationship between APD and CaT amplitude alternans was observable: larger APD alternans were associated with larger CaT amplitude alternans (n=15). Non-alternating AP sequence failed to induce CaT alternans, which finding assumes the possible role of transmembrane ionic currents (e.g. recovery kinetics of I_{CaL}).

4. DISCUSSION

Previous data on the Ca^{2+} dependent molecular mechanisms underlying the spontaneous activity of SAN cells are controversially discussed. Therefore the aim of this study was to investigate the assumed contribution of 1) forward NCX, 2) reverse NCX, 3) $I_{K(Ca)}$ in SAN automaticity and 4) to analyse the rate-dependent consequence of SAN pacemaking on the ventricular AP morphology.

4.1 Forward mode of the NCX has an important role in the spontaneous automaticity forming a strong functional coupling with I_f

A slight, but statistically significant decrease in the spontaneous AP firing rate was found in SAN tissue after selective NCX inhibition by ORM. The CL prolongatin effect of ORM is a direct experimental evidence proving the contribution of the inward depolarizing NCX current in the spontaneous rhythm generation. Yaniv et al. showed in cellular level that not only the frequency decrease, but the parallel increase of rhythm variablity reports the uncoupling of I_f-NCX cooperation and the destabilization of the DD. In our experiments the CL variability did

not change and the pacing rate was only slightly reduced, therefore we suggest that individual NCX inhibition does not result considerable uncoupling of I_f-NCX. Our results indicate that a functional coupling between NCX and I_f is present and is able to compensate for the NCX inhibition mediated reduction of the firing rate. The compensating reserve capacity between the currents could be the reason for the moderate effect of 3 μM or 10 μM ivabradine on CL.

Functional cooperation of I_f and NCX can create a phenomenon that is similar to the repolarization reserve in cardiomyocytes, and it can prevent the SAN cells from notable changes in the spontaneous frequency caused by individual inhibition of NCX or I_f. In the next set of experiment, the SAN firing rate was decreased by complete block of I_{Kr} that caused a statistically significant CL prolongation by enhancing the APD. In this case, NCX inhibition provided the same effect to the case when ORM was applied individually. The underlying reason could be the I_f dependent compensation of the NCX effect. Assuming that a strong cooperation exists between NCX and I_f, the "crosstalk" should work vica versa, i.e. the impairment of Ca^{2+} handling properties should affect I_f. In line with previous data, joint application of ryanodine and ORM caused ~20% increase in the CLs. Subsequent administration of ivabradine exerted considerably larger CL lengthening effect than in experiments where ivabradine was applied alone. Previous studies assumed that I_f-NCX coupling not only set the actual spontaneous firing rate but it may have an important role in the maintenance of the stable rhythm of SAN. Our experiments indicated that individual inhibition of NCX or I_f does not upset the rhythmicity of pacemaking. However, when both currents were reduced, besides the remarkable CL prolongation, a perturbation in the spontaneous rhythmicity also appeared resulting from the exhausted capacity of I_f-NCX coupling to depolarize the membrane.

Taking together, I_f and NCX contributes to DD forming a "pacemaker reserve". Similar to the concept of repolarization reserve, individual inhibition of NCX or I_f does not lead to excessive decrease of automaticity because the other, intact current is able to compensate for the effect of inhibition. This crosstalk could increase the robustness of pacemaking by providing a safety margin.

4.2 Reverse mode of the NCX contributes to pacemaking by refilling the Ca^{2+} clock

NCX equilibrium potential (calculated based on our experimental results) suggests a potential development of reverse NCX current in the first 55-65 ms of the AP. In line with this calculation, an outward $NiCl_2$ sensitive current was observed in this time range when

intracellular [Na^+] was 8 mM, but no outward current appeared when intracellular [Na^+] was reduced to 2 mM in the pipette. Similar result was obtained when ORM was applied. This result indicates that a $NiCl_2$- and ORM-sensitive and $[Na^+]_i$ dependent outward current is active during the first part of the SAN AP where the membrane potential is depolarized. This characteristic of the current suggests that reverse NCX exists during the SAN AP. Larger SR Ca^{2+} content and consequently larger Ca^{2+} transient amplitude was found in the presence of active reverse NCX. It suggests that active reverse mode provides an additional Ca^{2+} influx – supporting the I_{CaL} function – making the SR refilling a redundant mechanism that could improve the robustness of SAN pacemaking. An interesting further question was whether this additional, reverse NCX mediated Ca^{2+} influx has any role in setting the actual SAN CL. AP measurements with 2 and 8 mM [Na^+] in the pipette solution revealed that in the presence of active reverse mode the CL was shorter and the diastolic slope was steeper. Reverse NCX mediated Ca^{2+} influx provides an important fraction of total SR Ca^{2+} content, therefore it could contribute to fine tuning of the heart rate.

4.3 $I_{(KCa)}$ has no role in the spontaneous automaticity in basal conditions

When 8 mM [Na^+] was used in the patch pipette the SAN APD was shortened. This finding raises the possibility that other Ca^{2+} dependent mechanisms, such as Ca^{2+}-activated K^+-current could contribute to APD shortening. In contrast, we found no apamin sensitive current and in line with this, we failed to observe apamin induced frequency alteration and AP morphology change in SAN cells under normal conditions. Similar results were observed in a recent clinical trial. In a randomized, double-blind, placebo controlled phase I study by Gao et al., forty-seven healthy male volunteers were enrolled and examined after receiving $I_{K(Ca)}$ blocker AP30663 in single ascending dose[101]. No effect of AP30663 was seen on electrocardiographic parameters, such as RR interval, irrespective of the applied dose[101]. This finding is in line with our experimental results claiming negligible apamin sensitive current during the SAN action potential in response to dynamic intracellular Ca^{2+} changes under basal conditions.

4.4 Ventricular alternans are independent from restitution

In our tissue experiments it was found that the APD gradually shortened as pacing frequency increased, and when pacing rate reached a given threshold, APD alternans developed in all cases. At the same time, the development of APD alternans could be induced irrespective of the restitution slope, i.e. alternans appeared when restitution slope was steep or flat. This

result indicates negligible role of restitution curve in the prediction of alternans. A close relationship was found between APD and CaT alternans, supporting bidirectional coupling between membrane potential (i.e. action potential) and Ca^{2+} handling: when APD alternans were larger, CaT alternans were also enhanced. The magnitude of alternans of both variables was larger in the presence of higher pacing frequency.

Our results imply that alternans could be considered as a deleterious consequence of excessive SAN frequency. It is important to note that despite of the fact that alternans are frequency induced, under healthy condition the threshold frequency required for alternans induction is out of the range of physiological heart rate. However, in several diseases such as heart failure, the threshold frequency could be shifted toward lower frequencies. In this case alternans induction can occur via slightly increased heart rate that may cause life threatening arrhythmias.

5. CONCLUSION

In conclusion, it was found that I_f-NCX cooperation - in maintaining the diastolic depolarization - as well as the I_{CaL}-reverse NCX cooperation - in refilling the SR - may provide two parallel redundant systems to make the SAN pacemaking more robust, i.e. fail-safe. SAN pacemaking considerably influences the morphology of the ventricular APs and above a given frequency threshold it can induce alternans which are irrespective of restitution.

The main findings of this Ph.D. thesis are the followings:

1.) With the direct pharmacological inhibition of NCX, we provided experimental evidence regarding the role of forward NCX in the spontaneous pacemaking, furthermore, our data also supported a strong coupling between NCX and I_f. Individual inhibition of the currents does not lead to significant frequency changes and does not perturb the rhythmicity and stability of spontaneous automaticity. I_f and NCX may be able to develop a pacemaker reserve capacity, where they can compensate each other's operation ensuring stable pacemaking.

2.) In agreement with previous computational simulations[81] a voltage-, and $[Na^+_i]$ dependent outward current was found during the initial part of the SAN action potential which was sensitive to ORM and $NiCl_2$, indicating that the current is reverse NCX. Reverse NCX mediated Ca^{2+} influx contributes to SR Ca^{2+} refilling and facilitates SAN pacemaking.

3.) Negligible Ca^{2+} activated K^+ current was developed under the SAN action potential, indicating no function of the current in the rhythm generation in normal conditions.

4.) Restitution has no role in the prediction of APD and CaT alternans.

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