Characterization of different aspects of selective NCX inhibition in the heart: from inotropy to arrhythmias

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LIST OF PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Full length papers

I. Kohajda Z, Farkas-Morvay N, Jost N, Nagy N, Geramipour A, Horváth A, Varga RS, Hornyik T, Corici C, Acsai K, Horváth B, Prorok J, Ördög B, Déri Sz, Tóth D, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Baczkó I, Leprán I, Nánási PP, Papp JGy, Varró A, Virág L.
The effect of a novel highly selective inhibitor of the sodium/calcium exchanger (NCX) on cardiac arrhythmias in in vitro and in vivo experiments.
PLOS ONE 11(11): e0166041. doi: 10.1371/journal.pone.0166041.eCollection (2016)
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II. Nagy N, Kormos A, Kohajda Z, Szebeni A, Szepesi J, Pollesello P, Levijoki J, Acsai K, Virag L, Nanasi PP, Papp JGy, Varro A, Toth A
Selective Na^{+}/Ca^{2+} exchanger inhibition prevents Ca^{2+} overload induced triggered arrhythmias.
BRITISH JOURNAL OF PHARMACOLOGY 171(24):pp.5665-5681(2014)
IF: 4.842 (Q1/D1)

III. Jost N, Nagy N, Corici C, Kohajda Zs, Horváth A, Acsai K, Biliczki P, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Papp JGy, Varró A, Virág L.
ORM-10103, a novel specific inhibitor of the sodium/calcium exchanger, decreases early and delayed afterdepolarization in the canine heart.
BRITISH JOURNAL OF PHARMACOLOGY 170:(4) pp. 768-778. (2013)
IF: 5.067 (Q1/D1)

Quotable abstracts

Jost N, Zs Kohajda, N Nagy, A Geramipour, RS Varga, T Hornyik, B Horváth, A Kormos, K Acsai, J Levijoki, P Pollesello, T Koskelainen, L Otsomaa, A Tóth, I Baczkó, PP Nánási, JGy Papp, L Virág, A Varró.
Role of Na+/Ca2+ exchanger (NCX) in cardiac automaticity and ventricular repolarization assessed by ORM-10962, a novel highly selective NCX inhibitor
HEART RHYTHM, 13(5S), S6, 2016
IF (2015): 4.391

Nagy N, Kohajda Zs, Acsai K, Kormos A, Tóth A, Oravecz K, Pollesello P, Levijoki J, Virág L, Jost N, Papp JGy, Varró A
Inhibition and activation of the cardiac Na+/Ca2+ exchanger – two aspects of transport modulation
CURRENT RESEARCH: CARDIOLOGY - EXPERIMENTAL CLINICAL 2:(3) p. 143. (2015)

OTHER PAPERS

I. Major P , Baczko I , Hiripi L , Odening KE , Juhasz V , Kohajda Z , Horvath A , Seprenyi G , Kovacs M , Virag L , Jost N , Prorok J , Ordog B , Doleschall Z , Nattel S , Varro A , Bosze Z.
A novel transgenic rabbit model with reduced repolarization reserve: long QT syndrome caused by a dominant-negative mutation of KCNE1 gene.
BRITISH JOURNAL OF PHARMACOLOGY 173:(12) pp. 2046-2061. (2016)
IF: 5.259 (Q1/D1)

II. Ordog B , Hategan L , Kovacs M , Seprenyi G , Kohajda Z , Nagy I , Hegedus Z , Kornyei L , Jost N , Katona M , Szekeres M , Forster T , Papp JG , Varro A , Sepp R
Identification and functional characterisation of a novel KCNJ2 mutation, Val302del, causing Andersen-Tawil syndrome
CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 93:(7) pp. 569-575. (2015)
IF: 1.704 (Q2)

III. Corici C , Kohajda Z , Kristóf A , Horvath A , Virág L , Szél T , Nagy N , Szakonyi Zs , Fülöp F , Muntean DM , Varró A , Jost N
L-364,373 (R-L3) enantiomers have opposite modulating effects on I-Ks in mammalian ventricular myocytes
CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 91:(8) pp. 586-592. (2013)
IF: 1.546 (Q2)

IV. Jost N , Virág L , Comtois P , Ördög B , Szűts V , Seprényi Gy , Bitay M , Kohajda Zs , Koncz I , Nagy N , Szél T , Magyar J , Kovács M , Puskás LG , Lengyel Cs , Wettwer E , Ravens U , Nánási PP , Papp JGy , Varró A , Nattel S
Ionic mechanisms limiting cardiac repolarization-reserve in humans compared to dogs.
JOURNAL OF PHYSIOLOGY-LONDON 591:(17) pp. 4189-4206. (2013)
IF: 4.380 (Q1/D1)

V. Kristóf A , Husti Z , Koncz I , Kohajda Zs , Szél T , Juhász V , Biliczki P , Jost N , Baczkó I , Papp JGy , Varró A , Virág L
Diclofenac prolongs repolarization in ventricular muscle with impaired repolarization reserve.
PLOS ONE 7:(12) p. e53255. (2012)
IF: 3.730 (Q1)

VI. Jost N , Kohajda Z , Kristof A , Kovacs PP , Husti Z , Juhasz V , Kiss L , Varro A , Virag L , Baczko I
Atrial remodeling and novel pharmacological strategies for antiarrhythmic therapy in atrial fibrillation.
CURRENT MEDICINAL CHEMISTRY 18:(24) pp. 3675-3694. (2011)
IF: 4.859 (Q1)

VII. Virág L , Jost N , Papp R , Koncz I , Kristóf A , Kohajda Zs , Harmati G , Carbonell-Pascuall B , Ferrero JM , Papp JGy , Nánási PP , Varró A
Analysis of the contribution of I(to) to repolarization in canine ventricular myocardium.
BRITISH JOURNAL OF PHARMACOLOGY 164:(1) pp. 93-105. (2011)
IF: 4.409 (Q1/D1)

INTRODUCTION

1.1. The cardiac action potential and its underlying currents

The cardiac electrical cycle has been divided into five “phases”. The initial phase (phase 0) refers to the fast depolarization of the AP supported by the activation of fast inward Na^+ channels (I_{Na}). Beyond activating other currents of the AP (e.g.: Ca^{2+}-current, K^+-currents) this phase is responsible for the rapid impulse propagation. The phase 1 reflects a transient repolarization, mainly supported by transient outward potassium current (I_{to}). The magnitude of this phase has important role in shaping the spike-and-dome configuration of the AP. Since the expression level of ion channel(s) carrying I_{to} exerts marked differences across the ventricular wall (and therefore the amplitude of I_{to} as well) the spike-and-dome configuration can be considered a specific “marker” in identifying the ventricular origin of the cell. The phase 2 also named “plateau phase”, which is a characteristic feature of the cardiac AP. During this phase the inward and outward currents transiently balance each other, providing a long-lasting isoelectric phase, which has crucial role in cell contraction. Thus I_{CaL} is an important player not only in shaping the action potential, but in initiation of intracellular Ca^{2+} cycle. When I_{CaL} slowly decays, the outward K^+-currents overcome the charge influx, allowing repolarization (phase 3). During the initial section of phase 3, the rapid and slow components of delayed rectifiers (I_{Kr} and I_{Ks}) support large outward K^+-current having crucial role in repolarization, while in the terminal phase of the AP, the inward rectifier K^+ current (I_{K1}) has primary function in the complete repolarization. The phase 4 represents the resting membrane potential during diastole. Mainly I_{K1} and perhaps the Na^+/K^+ pump has important role in maintaining the stable resting membrane potential in ventricular cells. In atrial and Purkinje cells, where the expression level of I_{K1} is significantly smaller, the resting membrane potential is unstable, and a slow depolarization can be observable (diastolic depolarization), which has important role in the pacemaker function.

1.1.1. The concept and role of the repolarization reserve

The concept of the repolarization reserve was introduced by Roden, based on clinical observations. Based on clinical and experimental observation it was concluded that in the mammalian (human, dog and rabbit) ventricular muscle, inhibition of one type of potassium channels does not cause excessive APD lengthening. This is probably due to the capability of the various potassium channels which are able to substitute and/or supplement each other. Human, dog and rabbit ventricular myocytes seem to repolarize with a strong safety margin (“repolarization reserve”). This repolarization capacity has important role in decreasing the transmural APD dispersion, thus in preventing life-treating arrhythmias. When this normal repolarization reserve is attenuated due to drug exposures (cardiac and non-cardiac drugs as well), remodelling caused by diseases (heart failure, diabetes mellitus, hypothyroidism, etc), increased sympathetic activity, extreme bradycardia, hypokalaemia or genetic disorders (long QT syndromes, Brugada, etc), the otherwise minimal or moderate potassium current inhibition can result in excessive and potentially proarrhythmic prolongation of the ventricular

action potential duration. Multiple K^+ channel block can result in excessive repolarization lengthening by eliminating the repolarization reserve and therefore it can be associated with increased proarrhythmic risk.

1.1.2. Transmural heterogeneity in the ventricular myocardium

Studies described important electrophysiological and pharmacological regional differences of ventricular myocardium in mammalian heart. We can distinguish four functional cell types in the ventricles: epicardial, midmyocardial, endocardial and Purkinje cells. Epicardial and endocardial cells have shorter AP than midmyocardial cells. In epicardium the I_{to} and I_{Ks} are more abundantly expressed than in other layers. Midmyocardial cells (M cells) have intermediate electrophysiological features compared with Purkinje cells and its pharmacological responses are different from those of either epicardium or endocardium. Compared with the epicardium and endocardium, the M cells can be characterized by lowest I_{Ks} and highest I_{NaL} expression levels, pronounced frequency dependence of the APD, and marked effect of potassium inhibitions on repolarization. The M cells are well coupled electrically with the epicardium and endocardium to stabilize and compensate for the extreme APD lengthening. The I_{CaL}, I_{Kr} and I_{K1} are similar in the cardiac layers, but sodium/calcium exchanger (NCX) may exhibit the most abundant expression level in M cells.

1.2. Function of the sodium/calcium exchanger in the heart

1.2.1. Physiological role of NCX in ventricular myocardium

NCX is an important contributor to the Ca^{2+} homeostasis in the myocardium, having a crucial role in Ca^{2+} extrusion from the cell. The NCX is tightly regulated by the transmembrane Na^+ and Ca^{2+} gradients and by the actual level of the membrane potential. NCX can operate in a bidirectional fashion. In the forward mode NCX extrudes 1 Ca^{2+} from the cell which is coupled with 3 Na^+ ions entering the cell. Thus, the forward mode generates inward current, which eventually depolarizes the cell membrane. The reverse mode of the NCX is activated when the cytoplasmic Na^+ level is increased and the Ca^{2+} concentration is low and/or the membrane potential is depolarized. In this mode the NCX extrudes 3 Na^+ from the cell and moving 1 Ca^{2+} into the cell, and it can generate repolarizing net current. Under physiologic conditions, NCX operates mainly in forward mode and removes the same amount of Ca^{2+} that entered the cell through I_{CaL} maintaining the beat-to-beat Ca^{2+} balance. In the same time, Ca^{2+} elimination leads to Na^+ influx. In the excitation-contraction coupling (ECC), Ca^{2+} ions enter the cell from the extracellular space (Ca^{2+}-influx) mainly *via* I_{CaL}. The Ca^{2+} influx increases the Ca^{2+} concentrations near the ryanodine receptors (RyRs), which triggers Ca^{2+} release from the sarcoplasmic reticulum (SR). This mechanism is called 'Ca^{2+}-induced Ca^{2+} release' (CICR), which leads more Ca^{2+} to be released into the cytosol, resulting in a Ca^{2+} transient (CaT). In cardiac muscle CaTs can be observed as a summary of spatio-temporally restricted Ca^{2+} sparks, which can be monitored by optical (fluorometric) techniques. The free intracellular Ca^{2+} binds to the myofilaments and triggers contraction. After the contraction the released $[Ca^{2+}]_i$ is eliminated by both Ca^{2+} reuptake

to the SR *via* the activity of the SR Ca^{2+} pump (SERCA2a), and Ca^{2+} extrusion (efflux) from the cell (primarily by the forward mode activity of the NCX). In steady state, during each cycle, the released and reuptake Ca^{2+}, as well as the entered and extruded Ca^{2+} must be equal.

In the past two decades several NCX inhibitors were developed having different potency and selectivity. The KB-R7943 markedly suppressed the NCX current however exerted poor selectivity. The SEA0400 is a widely used, potent NCX inhibitor which considerably improved our knowledge about the NCX function however a ~ 20% inhibition of I_{Ca} made the data interpretation difficult. The ORM-10103 and ORM-10962 are recently synthesised, novel NCX inhibitors having an appropriate selectivity profile to investigate the NCX function (for details see the chapter 1.3). Theoretically, the inhibition of NCX may lead to net gain of the intracellular Ca^{2+} and causes positive inotropy. This effect may have important clinical implication since during heart failure (HF), the Ca^{2+}content of the cells is decreased thus the pump function is reduced. However, the possible positive inotropic effect of selective NCX inhibition remained controversial in the literature. Studies performed on rat myocytes and Langendorff perfused heart experiments reported clear positive inotropic effect of SEA-0400 however SEA0400 and ORM-10103 failed to influence the Ca^{2+} transients in dog ventricular cells. The controversial results may be caused by the incomplete selectivity of the applied drugs and/or interspecies differences, especially in the kinetics of the action potentials and the intracellular ion levels of the cells.

1.2.2. Possible role of NCX in the pacemaker tissue

The sinoatrial node (SA node) is the primary pacemaker in the heart generating regular, spontaneous APs, to initiate the normal cardiac cycle. Pacemaker potentials are often referred as "slow response" action potentials suggesting relatively low kinetics of the I_{CaT} and I_{CaL} during depolarization. The upstroke of the AP is preceded by a slow diastolic depolarization (DD), which slowly depolarizes the membrane potential to reach the excitation threshold. Any impact, which changes the slope of DD, will have an effect on cycle length and heart frequency. Recent works in the atrioventricular node and Purkinje cells suggest that the mechanisms governing sino-atrial node automaticity are likely to be similar in all pacemaking tissues. During spontaneous pacemaking, several ion channels (I_f, I_{CaT}, I_{CaL}, I_K) with finely tuned kinetics cooperate during an action potential waveform, this collective behaviour has been termed as “the membrane clock”. Dynamic time- and voltage-dependent interaction of the above membrane-limited electrogenic ion channels leads DD from MDP to threshold potential. Membrane clock interacting with the intracellular Ca^{2+} cycling transport mechanism establishes the “Ca^{2+} clock”. The main phenomenon is the spontaneous, periodic local subsarcolemmal Ca^{2+} release (LCRs) from SR during DD in pacemaker cells. These 1-5Hz LCRs activate NCX forward mode, and Ca^{2+} extrusion due to Na^+ influx making a depolarizing current. This current may accelerate the DD and contribute to spontaneous pacemaking. The two clocks may work synergistically forming a coupled-clock system. Previous studies suggested the pivotal role of the NCX in normal automaticity. The low-sodium containing bath solution inhibited spontaneous AP firing in guinea-pig SA node cells

via suppressing normal function of NCX. Other study reported that depletion of SR store by application of ryanodine markedly disturbed the normal pacemaker activity in rabbit SA node cells. However, these interventions may have effects on I_f and I_{CaL} directly, which makes the interpretation difficult. Mouse genetic models revealed that partial atrial NCX1 knock out (~90%) caused severe bradycardia and other rhythm disorders, while complete atrial NCX knock-out completely suppressed the atrial depolarization exerting ventricular escape rhythm on the ECG. The application of KB-R7943, a non-selective NCX inhibitor, also suppressed spontaneous beating in guinea-pig SA node cells; however, it has also effect on the Ca^{2+}-currents. The supposed crucial role of NCX in the normal pacemaker function of SA node cells could not be directly challenged experimentally so far, by the lack of selective NCX inhibitor.

1.2.3. Pathophysiological role of NCX in Na^+ induced Ca^{2+} load

The actual function of the NCX is tightly regulated by the intracellular levels of the Na^+ and Ca^{2+} ions. Therefore, any disturbances, which shift the normal balance of Na^+/ Ca^{2+} handling, will influence the NCX function. The altered function of NCX may lead to membrane potential instability and development of delayed afterdepolarizations. Several diseases which increase the level of intracellular Na^+ by reduction of the activity of Na^+/K^+ pump function (hypoxic conditions or digitalis intoxication) or by increasing the I_{NaL} (long QT Syndrome 3 – LQT3) can lead to marked activation of reverse mode NCX function, which accounts for the concomitant Ca^{2+} accumulation and the subsequent abnormal automaticity. It is suggested that selective NCX inhibition by suppressing either the reverse or the forward mode may have antiarrhythmic effect during Na^+ induced Ca^{2+} load. The antiarrhythmic effect of selective NCX inhibition is controversial in the literature. NCX inhibitors have shown antiarrhythmic effects in hearth rhythm disturbances evoked by ischemia/reperfusion injury *in vivo*, in Langendorff perfused hearts, and in pharmacologically simulated ischemia/reperfusion models. The SEA0400 decreased the incidence, and reduced the development of EADs, it failed to suppress the aconitine induced arrhythmias. In Langendorff-perfused rat hearts SEA0400 even enhanced the arrhythmia incidence and duration. The related studies from our laboratory are also contradictory. SEA0400 did not decrease QTc after dofetilide administration, and failed to prevent the development of Torsades de Pointes tachyarrhythmias (TdPs) in Langendorff-perfused rabbit hearts, while in another study it effectively reduced the amplitudes of EADs, without influencing APD. In contrast, Milberg et al reported considerable APD shortening effect of SEA0400, furthermore, sotalol or veratridine induced TdPs were also suppressed. Recently Jost et al. claimed that ORM-10103, a novel NCX inhibitor with improved selectivity decreased pharmacologically induced DADs and EADs, confirming previous results performed with SEA0400.

1.3. Summary of NCX inhibitors

Benzyloxyphenyl derivative inhibitors like KB-R7943, SEA0400, SN-6 were used successfully in several NCX studie. These compounds inhibit NCX from the external side. KB-R7943

was the first as a prototype in this NCX inhibitor family, which preferentially inhibits the reverse mode operation of NCX. In cardiac cells the reverse mode blocking effect was larger compared with the forward mode (EC_{50}= 0.3 µM on reverse *vs*. 17 µM on forward mode). SEA0400 is a much more potent NCX blocker (EC_{50}=111 nM on the reverse *vs*. 108 nM on the forward mode, , but both KB-R7943 and SEA0400 were shown to exert substantial nonspecific effects on several ion channels: I_{Na}, I_{CaL}, I_{K1} and delayed rectifier K^+ currents. SN-6 was developed from KB-R7943, but it also more potently inhibits outward, than inward NCX current (EC_{50}= 1.9 µM *vs* 2.3 µM) and also significantly suppresses other currents (I_{Na}, I_{CaL}, I_{Kr}, I_{Ks}, I_{K1}), causing AP shortening. The exchanger inhibitory protein (XIP) was developed as NCX inhibitor protein, interacting with the Na^+ regulatory domain of the NCX and suppresses both transport modes. However, the XIP fails to penetrate through the sarcolemma, so it can be used only intracellularly in patch clamp experiments. Recently, novel promising NCX inhibitors, ORM-10103 and ORM-10962 have been developed. The ORM-10103 inhibited both modes of the NCX, and successfully suppressed *in vitro* the pharmacologically induced EADs and DADs in dog heart preparations. With the exception of a 25.4% inhibition of the I_{Kr}, the ORM-10103 did not influence the major ionic currents. More recently, a novel compound, the ORM-10962 was synthesized exerting considerably lower EC_{50} levels with promising selectivity and antiarrhythmic profile.

1.4. Aims of the study

Since our knowledge about the role of NCX in the cardiac repolarization and in pacemaker activity is not fully clarified because of the lack of selective inhibitors, the aims of the present study were:

1) To analyse the effects of the newly synthesized NCX blockers ORM-10103 and ORM-10962 on the NCX current
2) To investigate the selectivity of ORM-10962 on major transmembrane ionic currents comparing with the results of ORM-10103
3) To study the effect of selective NCX inhibition on cardiac ventricular action potentials and on calcium transients
4) To investigate the role of NCX in the cardiac pacemaker function
5) To examine the effect of NCX inhibition on *in vitro* triggered arrhythmias

3. RESULTS

3.1. Inhibition of NCX current by ORM-10103 and ORM-10962

ORM-10103 applied at 10 µM concentration caused a comparable, significant inhibition of NCX both in the reverse as well as in the forward mode operation (82±9% and 88±6% inhibition, respectively, n=6 cell/2 hearts). The estimated EC_{50} values for the inward and outward NCX currents were 780 nM, and 960 nM, however, a small but statistically significant inhibition on the I_{Kr} indicated the importance of development of novel NCX inhibitor compounds with better selectivity profile and lower EC_{50} values. Therefore, we aimed to analyse the recently synthesized NCX inhibitor compound ORM-10962. ORM-10962 considerably decreased both the outward and the inward NCX current in a concentration-dependent manner. The effect of the drug at different concentrations on the outward NCX current (reverse mode) was calculated at 20 mV and on the inward current (forward mode) was determined at -80 mV. The estimated EC_{50} values of ORM-10962 for the outward and inward NCX currents were found to be 67 nM, and 55 nM, respectively.

3.2. Selectivity of ORM-10962

3.2.1. The effect of ORM-10962 on the L-type inward calcium current

The possible effect of ORM-10962 on the I_{CaL} was investigated in single dog ventricular myocytes. The following protocol was used for patch clamp measurements: the holding potential was -80 mV and a short prepulse to -40 mV was added to inactivate I_{Na}. I_{CaL} was activated by 400 ms long depolarizing voltage pulses to different test potentials ranging from -35 mV to 55 mV. These experiments clearly revealed that ORM-10962 even at high (1 µM) concentration did not influence I_{CaL}. The effect of ORM-10962 was also studied on slow response action potentials recorded from guinea-pig papillary muscles. ORM-10962 at 1 µM did not affect the amplitude or dV/dtmax of the slow response action potentials, which may support the results of the direct I_{CaL} measurements. In the same preparations for positive control, we used a well-established I_{CaL} blocker nisoldipine at 100 nM, which markedly reduced both the amplitude and dV/dtmax of the slow response action potentials.

3.3.2. The effect of ORM-10962 on late and peak sodium current, and on Na^+/K^+ pump currents

The late sodium current (I_{NaL}) was activated by depolarizing pulses to -20 mV from a holding potential of -120 mV. Addition of 1 µM ORM-10962 did not decrease the amplitude of I_{NaL} while 20 µM TTX completely blocked the current.

The peak sodium current ($I_{Na,peak}$) was measured in Nav1.5 overexpressed CHO cells at -20 mV depolarizing pulses from a holding potential of -90 mV. After applying 1 µM ORM-10962 the control $I_{Na,peak}$ was not significantly changed however, after 20 µM TTX the current markedly reduced.

The Na^+/K^+ pump current (I_p) was measured as a steady-state current at -30 mV. In control I_p decreased at the end of the 5–7 min incubation with 1 µM ORM-10962. A time control was made in a

separate set of experiments when the same protocol was applied with the vehicle of the ORM-10962 (DMSO). Similar small current decrease was recorded in the steady-state current, which was observed in the presence of the ORM-10962. Therefore, it is concluded that ORM-10962 does not influence Ip, the slight tendency of the current to decrease is not due to the effect of ORM-10962. For positive control 10 µM strophantin was used, which effectively suppressed the current and subsequent addition of 0 mM K^+ solution failed to cause further decrease in the Na^+/K^+ pump current justifying the complete inhibition of the current after strophantin application.

3.2.3. The effect of ORM-10962 on outward potassium currents

The effect of ORM-10962 on I_{K1}, I_{to}, I_{Kr} and I_{Ks} were also investigated. The I_{K1} was measured by determining the steady-state current-voltage relationship of the membrane at the end of 300 ms long pulses clamped to potentials ranging from -80 to 0 mV. I_{to} was activated by 1000 ms long depolarizing voltage pulses arising from the holding potential of -80 mV to test potentials gradually increasing up to 50 mV. I_{Kr} and I_{Ks} were determined as tail current amplitudes at -40 mV after activating these currents by 1000 ms (I_{Kr}) or 5000 ms (I_{Ks}) long depolarizing voltage pulses at various test potentials ranging up to 50 mV. It was found that neither of these currents was significantly influenced by 1 µM ORM-10962.

3.2.4. Selectivity of ORM-10962 on the pacemaker current

The effect of ORM-10962 on the I_f was investigated in rabbit right atrial cells isolated from the SAN region. The current was activated by hyperpolarizing voltage pulses to -120 mV from the holding potential of -30 mV. The pacemaker current was identified as ivabradine sensitive current. At negative hyperpolarizing membrane potential (from -30 mV to -120 mV) an ivabradine (10 µM) sensitive current could be measured, which was not altered after application of 1 µM ORM-10962.

3.3. Comparison of the effects of ORM-10103 and ORM-10962 on the major repolarizing transmembrane potassium currents

The I_{K1}, I_{to}, I_{Ks} did not show significant change after application either of 10 µM ORM-10103 or 1µM ORM-10926. In contrast, 10µM ORM-10103 significantly decreased the I_{Kr} amplitude; however, 1µM ORM-10962 did not change the current.

3.4. Effect of selective NCX inhibition on Ca^{2+} transient and action potentials under normal condition

The amplitude of Ca^{2+} transients and cell shortening slightly but statistically significantly increased after the addition of 1µM ORM-10962. The diastolic Ca^{2+} did not change significantly during the experiment. The characteristic of the AP did not change after application of 1 µM ORM-10962.

3.5. Effect of selective NCX inhibition on endocardial, epicardial tissues and on Purkinje fibres

In subendocardial multicellular preparation, we observed a moderate increase in the APD parameters after application of 1µM ORM-10962, however in subepicardial preparation the APD increased significantly. The Purkinje fibre preparations exerted inconsistent and negligible change in the action potential duration, where a negative shift of the plateau voltage was observed, without any significant changes in the repolarization parameters.

3.6. Effect of NCX inhibition on the spontaneous automaticity

3.6.1. Effect of NCX inhibition on spontaneous automaticity on Purkinje fibres and on atrial tissue

In dog Purkinje fibres 1 µM ORM-10962 exerted clear lengthening effect on the cycle length of spontaneous action potentials. In rabbit Purkinje fibres we observed significant cycle lengthening effect of spontaneous action potentials after application of 0.5 µM and 1 µM ORM. In rabbit atrial measurements, following application of 1µM ORM-10962 moderate but significant lengthening effect on the cycle length was observed. The corresponding time control measurements exerted no effect after application of the vehicle of ORM-10962 (DMSO).

3.6.2. Effect of NCX inhibition on spontaneous automaticity after ivabradine treatment

In this set of experiments 3 µM ivabradine was applied on rabbit right atrial tissue strips to reduce spontaneous frequency. The cycle length increased after application of 3 µM and 1 µM ORM-10962 caused further statistically significant increase. ORM-induced increase in the cycle length after application of ivabradine was significantly larger compared with the ORM application alone. During time control experiments, we found similar effect of ivabradine compared with control, but the subsequently applied vehicle (DMSO) failed to influence the cycle length.

3.7. The antiarrhythmic effect of selective NCX inhibition on delayed afterdepolarizations *in vitro*

In dog cardiac Purkinje fibres DAD was evoked by 150 nM digoxin, which is a well-known Na^+/K^+ pump inhibitor. After 40 stimuli train with a cycle length of 400 ms, several DAD's were observed during the stimulation-free period. Further addition of 1 µM ORM-10962 significantly decreased the DAD's amplitude. In some of these experiments digoxin evoked a run of extra beats (cellular, corresponding to the *in vivo* extrasystole) after the termination of the stimulus train, which could be successfully abolished by the application of 1 µM ORM-10962.

3.8. Effect of selective NCX inhibition on Ca^{2+} transient and action potentials when forward or reverse mode is facilitated

In the first set of experiment, extracellular Na^+ concentration was decreased to 70 mM, while the solution was supplemented with 70 mM choline-cloride thus the lower electrochemical driving force for Na^+ stimulates the reverse mode of NCX. In these measurements NCX inhibition with 1µM

ORM-10962 slightly, but significantly lengthened the repolarization of action potentials. The CaT and cell shortening increased by the administration of low Na-Tyrode, and significantly decreased when 1 µM ORM-10962 was applied. The diastolic Ca^{2+} significantly decreased after ORM-10962 application (*vs* low Na^{+}).

In another set of experiment, enhanced NCX forward mode was achieved by 1 µM forskolin which increases the intracellular Ca^{2+} level through protein-kinase mediated phosphorylation of the I_{CaL}. The plateau voltage of the action potential was significantly depressed after application of 1µM ORM-10962 without consistently changing phase 3 repolarization. Application of 600 nM forskolin in single ventricular dog myocytes increased the amplitude of transient and cell shortening and addition of 1 µM ORM-10962 increased further the amplitudes significantly. The diastolic Ca^{2+} did not change significantly during the experiment.

4. DISCUSSION

4.1. ORM-10962 exerted improved efficacy and specificity in comparison with ORM-10103

Previously, the most successfully used NCX inhibitors like KB-R7943 and SEA0400 considerably improved our knowledge about the role of NCX regarding the electromechanical coupling and arrhythmogenesis, but these inhibitors were not appropriately selective *i.e.* certain amount of I_{CaL} and I_K currents contaminated the results making the data interpretation difficult. Therefore, the most important requirement for a NCX inhibitor – beyond potent blocking of NCX – is a minimal influence on the I_{CaL} current. In previous studies of our laboratory, we reported that ORM-10103 effectively and selectively inhibits NCX in dog ventricular myocytes without influencing the I_{CaL} current. Furthermore it was effective against triggered arrhythmias, namely it clearly decreased the amplitude of pharmacologically induced EAD and reduced the DAD incidence. However, this compound had 25.4% blocking effect on I_{Kr} at close to the maximal NCX inhibition concentration range. Therefore, this compound has some limitations in studying the role of NCX since it also influences the ventricular repolarization. The improved compound, ORM-10962 has no effect on I_{CaL} even in higher concentration (1µM) as well as it does not influence the peak and late sodium currents and the N^{+}/K^{+} pump, and the main repolarizing potassium currents like I_{K1}, I_{to}, I_{Kr} and I_{Ks} at close the maximal NCX inhibition (~80%) concentration range. Furthermore, it has much less EC_{50} values (55/67nM for inward/outward NCX currents, respectively) at both modes of NCX. The better selectivity and improved efficacy of ORM-10962 account for performing the further experiments with ORM-10962.

4.2. Selective NCX inhibition has a moderate positive inotropic effect without major influence on the action potential under normal condition

It is widely accepted that the NCX operates primarily in forward mode during a normal AP, therefore, when inhibited, intracellular Ca^{2+} load, and AP shortening is expected. However, our laboratory and others failed to find any influence of SEA0400 and ORM-10103 on AP and CaT. In contrast, we observed a marginal but significant increase on CaT and cell shortening after application of ORM-10962 in dog ventricular myocytes. Thus, our results support the hypothesis regarding the positive inotropic effect of selective NCX inhibition; however, the relatively small extent of the effect is unexpected and unclear. A possible explanation could be the concomitant inhibition of reverse mode activity which simultaneously decreases the intracellular Ca^{2+} influx. Further possibility could be the PMCA, which may also contribute to Ca^{2+} extrusion, and therefore it may compensate for the reduced NCX function. Based on our previous study, where the NCX was measured in the presence of intact Ca^{2+} cycle, we proposed that under normal condition, the inward NCX current has relatively low amplitude thus the inhibition of this small current may be fully compensated by the above mentioned mechanisms. The effect of ORM-10962 on AP was investigated in multicellular preparations from different transmural regions and showed tissue specific changes. The selective NCX inhibition marginally increased the APD_{90} in epicardial tissue without significant influence on subendocardial region and in Purkinje fibres. In healthy myocardium the cells are well coupled electrically, and this electronic interaction largely minimizes the repolarization changes at tissue level, while these alterations could be augmented at cellular level which may explain the marginal effect in epicardial tissue. Previous studies seem to support our suggestion that under normal condition, only small net NCX current is evoked during the AP and the influence of the inhibition of this small current may be compensated for by the repolarization reserve. The momentary magnitude of NCX current under the AP depends on the actual voltage, intracellular Na^{+} and Ca^{2+} levels. The kinetics of the APs, CaTs may differ regarding the tissue type (subendocardial, subepicardial, Purkinje fibre) and could also be different from cell to cell obtained from the same heart region. We cannot rule out the possibility that the net current of the NCX is changing throughout the ventricular wall: it becomes inward from epi- to endocardial tissue.

4.3. Selective NCX inhibition decreases the spontaneous pacing rate of SA node and Purkinje fibres under normal conditions

We found significant negative chronotropic effect of ORM-10962 in the SA node and Purkinje fibres. Previous studies showed that two competing mechanisms may play a role during the initiation of each heartbeat: one is the M (membrane voltage) clock claiming pivotal role of I_f in determining the actual frequency, and the second one is the Ca^{2+} clock. According to the Ca^{2+} clock hypothesis, the local Ca^{2+} release from the SR causes local depolarization, which enhances the NCX forward activity. Forward NCX due to Ca^{2+} extrusion generate depolarizing Na^{+} influx, thus may contribute to the

gradual depolarization initiated by the I_f pacemaker current. Our results seem to be in line with this hypothesis, since NCX inhibition by ORM-10962 decreased the spontaneous firing rate in both canine and rabbit Purkinje-fibres as well as in rabbit atrial tissue samples. Therefore, NCX inhibition may suppress the spontaneous diastolic depolarization and increase the cycle length of the pacemaker cells, without directly influencing the I_f . However, considering these marginal but statistically significant effect of ORM-10962, we assume that the NCX may have only a small modulatory role in diastolic depolarization. The current theory synthesizes the M-clock and Ca-clock mechanisms, claiming Ca^{2+}-clock together with M-clock form a coupled-clock system where neither clock is dominant; instead together they control the spontaneous AP firing frequency and rhythm in the pacemaker cells. These studies showed that ivabradine, however inhibits selectively the I_f current, it also indirectly suppresses Ca^{2+} cycling by reducing SR Ca^{2+} load, without direct influence on the ion channels (I_{Ca}, RyR) of the Ca^{2+} handling. These results may indicate that the bradycardia occurred by ivabradine is not only the consequence of the I_f blockade alone, but it is rather due to the perturbation of the crosstalk within the coupled-clock system. The underlying mechanism may be the reduction in SR load, which leads to prolonged local Ca^{2+} release period, causing reduced and delayed NCX function in the membrane clock. Our results may support this crosstalk theory since the AP firing frequency of the ivabradine pre-treated spontaneous rabbit atria was further reduced after application of ORM-10962.

4.4. NCX inhibition is effective against Na^+ induced Ca^{2+} load mediated delayed afterdepolarizations

Previous studies with SEA0400 and ORM-10103 suggested that NCX inhibition could be effective against Na^+ induced Ca^{2+} load mediated DADs. The afterdepolarizations were evoked by inhibition of Na^+/K^+ pump by glycosides, leading to accumulation in $[Na^+]_i$ and AP shortening. The elevated intracellular Na^+ level induces increased reverse NCX activity causing marked gain in intracellular Ca^{2+} and consequently in the SR Ca^{2+} level. Selective NCX inhibition by applying ORM-10962 completely abolished the digoxin induced spontaneous automaticity and significantly decreased DADs amplitude. Similar results were observed by application of ORM-10103, where not only DAD, but also EAD amplitudes reduced significantly after the ORM-10103 treatment, however this compound is not completely selective for NCX. The underlying mechanism of the effect is not exactly clarified. During Na^+ induced Ca^{2+} load both modes of the NCX is facilitated by the increased level of the Na^+ and Ca^{2+} ions. Therefore, theoretically inhibition of both modes could be antiarrhythmic since suppression of the reverse mode may decrease the intracellular Ca^{2+}, while inhibition of forward mode may directly reduce the amplitude and incidence of DAD's amplitude. In order to address this question we attempted to selectively facilitate the reverse and mode NCX activity. In the presence of low Na^+ containing Tyrode's solution, clear negative inotropic effect could be observed after application of ORM-10962 suggesting a decrease in $[Ca^{2+}]_i$ level. Furthermore, the APD was marginally but statistically significantly lengthened which may also indicate increased reverse mode activity and

consequently larger outward current. This result may also suggest that during Na^+ induced Ca^{2+} load the NCX operates mainly in reverse mode therefore its inhibition resulted in net loss of intracellular Ca^{2+} and negative inotropy. Similar results were obtained with ORM-10103 in our previous study.

In a separate experiment, the forward mode was facilitated by forskolin which increases the intracellular Ca^{2+} level via adenylate-cyclase activation. Under this setting the selective NCX inhibition caused net gain in intracellular Ca^{2+} and positive inotropy as well as slight depression in the AP plateau potential. The positive inotropy can be explained by the shift of the NCX reverse potential caused by increased intracellular Ca^{2+} which may result in shift to the positive potentials facilitating ion transport in the forward mode. The inhibition of the NCX may reduce the rate of the Ca^{2+} extrusion with a concomitant compensatory reduction of the I_{CaL} (the Ca^{2+} influx and efflux must be equal) achieved by the increased Ca^{2+} transient. This shift in current balance may account (at least partially) for the depressed plateau level of the action potential.

Summarizing these results we suggest that during Na^+ induced Ca^{2+} load both modes of the NCX is facilitated but the reverse mode may be predominant during this condition. Inhibition of both modes could be theoretically antiarrhythmic. However, the inhibition of the reverse mode may have more importance since the net loss of the intracellular Ca^{2+} may indirectly reduce the forward mode activity and *per se* reduces the DAD amplitude and incidence.

5. CONLUSIONS AND POTENTIAL SIGNIFICANCE

1) We have shown that two newly synthesized NCX inhibitors, ORM-10103 and ORM-10962, effectively inhibit both modes of the NCX current. ORM-10962 is more potent than the previously described ORM-10103, having the EC_{50} value of the ORM-10962 in the nanomolar range.
2) ORM-10962 was found a highly selective NCX inhibitor, because even a higher concentration (1μM) did not influence other transmembrane ionic currents in the heart (I_{CaL}, I_{NaL}, $I_{Na\ peak}$, Na^+/K^+ pump, I_{Kr}, I_{Ks}, I_{to}, I_{K1}, I_f). However, the ORM-10103 in a higher concentration (10 μM) has a moderated I_{Kr} blocking effect.
3) Selective NCX inhibition by ORM-10962 exerted a marginal positive inotropic effect as shown by CaT and cell shortening measurements. In the same time, it failed to influence the ventricular AP under normal condition.
4) In line with previous hypothesis we have demonstrated experimentally that NCX has a role in the pacemaking mechanisms. The NCX inhibition significantly prolongs the cycle length of spontaneous APs recorded from dog and rabbit Purkinje fibres and atrial samples. Moreover, addition of ORM-10962 after ivabradine application a further reduction in the spontaneous AP firing frequency was observed which may support the coupled-clock hypothesis.
5) NCX inhibition completely abolished the digoxin-induced automaticity and significantly decreased the amplitude of DAD's. The underlying mechanism was addressed by investigating the shifts in the balance of the forward and reverse modes of NCX. When forward mode was facilitated, NCX inhibition caused a significant positive inotropic effect without major change in APD, however in those experiments in which reverse mode was enhanced negative inotropic effect was observed, with APD lengthening. We conclude that under Na^+ induced Ca^{2+} load the reverse mode became predominant therefore the subsequently applied NCX inhibition decreases the Ca^{2+} transient amplitude. In line with this, we suggest that during digoxin-induced DADs, where also Na^+ induced Ca^{2+} load occurred, the inhibition of the facilitated reverse mode may have more importance in the development of antiarrhythmic effect.

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