The inotropic consequences of selective Na^+/Ca^{2+} exchanger inhibition is controlled by the actual transport balance

PhD Thesis

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PUBLICATIONS

Full length papers related to the thesis

I. Oravecz K, Kormos A, Gruber A, Márton Z, Kohajda Z, Mirzaei L, Jost N, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Papp JG, Nánási PP, Antoons G, Varró A, Acsai K, Nagy N.
Inotropic effect of NCX inhibition depends on the relative activity of the reverse NCX assessed by a novel inhibitor ORM-10962 on canine ventricular myocytes.
Eur J Pharmacol. 2018 Jan 5;818:278-286

II. Geramipour A, Kohajda Z, Corici C, Prorok J, Szakonyi Z, **Oravecz K**, Márton Z, Nagy N, Tóth A, Acsai K, Virág L, Varró A, Jost N.
The investigation of the cellular electrophysiological and antiarrhythmic effects of a novel selective sodium-calcium exchanger inhibitor, GYKB-6635, in canine and guinea-pig hearts.
Can J Physiol Pharmacol. 2016 Oct;94(10):1090-1101

III. Prorok J, **Oravecz K**, Gazdag P, Frey Zs, Vígh D, Nagy N, Acsai K, Tóth A, Jost N, Papp JGy, Varró A
Selective inhibition of the NCX attenuates the hypokalaemia-induced elevated intracellular Ca^{2+} load and decreases the incidence of ventricular arrhythmias
Manuscript in preparation

Study not related to the subject of the thesis

I. Acsai K, Nagy N, Marton Z, **Oravecz K**, Varro A.
Antiarrhythmic potential of drugs targeting the cardiac ryanodine receptor Ca^{2+} release channel: case study of dantrolene.
Curr Pharm Des. 2015;21(8):1062-72. Review.

ACRONYMS AND ABBREVIATIONS

AP: action potential

APD: action potential duration

ATP: adenosine-triphosphate

AU: arbitrary unit

CaM: calmodulin

cAMP: cyclic adenosine-monophosphate

CDI: Ca^{2+} dependent inactivation

CICR: Ca^{2+} induced Ca2+ release

cAMP: cyclic adenosine-monophosphate

CDI: Ca^{2+} dependent inactivation

CaT: Ca^{2+} transient

CS: Cell shortening

DAD: delayed afterdepolarizations

DD: diastolic depolarization

DIDS: 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid

EAD: early afterdepolarizations

ECC: excitation-contraction coupling

$[Ca^{2+}]_i$: intracellular Ca^{2+}

GYKB-6635: ((4-amino-1-[((1R,2S,3S,5R)-2,3-dihydroxy-6,6-dimethylbicyclo[3.1.1]heptan-2-yl)-methyl] pyrimidin-2(1H)-one)

I_{CaL}: L-type Ca^{2+} current

I_{CaT}: T-type Ca^{2+} current

I_{Na}: Na^+ current

I_{NaL}: Late Na^+current

I_{NaK}: Na^+/K^+ pump current

NCX: Na^+/Ca^{2+} exchanger

NKA : Na^+/K^+ ATPase

ORM-10103 : NCX inhibitor

ORM-10962: NCX inhibitor

PMCA : sarcolemmal Ca^{2+} ATPase

RyR: ryanodine receptor

SERCA2a: myocardial sarcoplasmic reticulum Ca^{2+} ATPase

SR: sarcoplasmic reticulum

INTRODUCTION

Cardiovascular diseases are associated with high mortality rate, which challenges both the current research activity and the healthcare budget. Since the conventional antiarrhythmic drugs used in the treatment of heart failure have serious side effects there is an increasing demand about new drug targets as well as novel selective inhibitors. One of a prosperous new drug target is the Na^+/Ca^{2+} exchanger which has pivotal role in several arrhythmias, Nevertheless selective inhibitor has not been available so far. However the recently synthesized, selective compounds may provide novel perspectives in the future drug therapy.

Intracellular calcium homeostasis of the heart

The Ca^{2+} homeostasis of the cardiac cells is a fine tuned intracellular circulation of the Ca^{2+} ions establishing a connection between the sarcolemmal voltage changes and myocyte contraction. This function of the Ca^{2+} handling is the excitation-contraction coupling (ECC) which is designed to provide an effective and adaptive cardiac output by the activation of myofilaments during various circumstances. The ECC is tightly controlled by a complex and precise mechanism initiated by quick depolarization of the sarcolemma and finished by the contraction-relaxation cycle. During the ECC, Ca^{2+} ions enter the cell from the extracellular space (*Ca-influx*) than trigger a large *Ca^{2+} release* from the sarcoplasmic reticulum (SR). The elevation of Ca^{2+} transient (CaT) is ceased by the *Ca^{2+} reuptake* to the sarcoplasmic reticulum, (via the activity of SR Ca^{2+} pump, $SERCA_{2a}$) and on the other hand by the Ca^{2+} extrusion (*Ca-efflux*) from the cell, (primarily via the forward mode activity of the NCX). In the first section of this thesis a brief overview is provided about the above mentioned sections of the Ca^{2+} handling.

Calcium influx

The Ca^{2+} influx as an initiator process of the Ca^{2+} handling includes different mechanisms by which the Ca^{2+} is able to enter into the cell. However the reverse mode of the Na^+/Ca^{2+} exchanger as well as Na^+ channels may also contribute to the Ca^{2+} influx the main mechanism of which is the Ca^{2+} transport through different Ca^{2+} channels. Two main subtypes of the Ca^{2+} channels are expressed in cardiac myocytes: the T (transient) and L (long-lasting) types. The T-type channels are primarily expressed in the nodal tissue (sinoatrial node, atrioventricular node) and the atrial cells, and their role in the ventricular cells may be negligible. In contrast, L-type channels are abundantly expressed at the sarcolemmal junctions of ventricular cells. L-type Ca^{2+} channels show a large conductance during depolarization, (in more positive activation range compared with T-type) therefore a small amount of Ca^{2+} flux

which enters the cell can release an extensively high Ca^{2+} flux from the SR, via triggering the ryanodine receptors (RyR). This above mentioned mechanism is called Ca^{2+} induced Ca^{2+} release (CICR). The reverse operation mode of the NCX, primarily at the beginning of the action potential, passes a minor Ca^{2+} flux to the cell which is counterbalanced by Na^{+} extrusion. Ca^{2+} influx and the amount of the entering ions are controlled by the magnitude and the time course of SR Ca^{2+} release by a negative feedback mechanism which is called Ca^{2+} dependent inactivation (CDI) .

Calcium release

The main function of the sarcoplasmic reticulum is to store a large amount of Ca^{2+}, mostly bounded to a Ca^{2+} binding protein calsequestrin. When the trigger Ca^{2+} reaches the RyR, the conductance of RyR is elevated facilitating the fast release of the stored Ca^{2+}. Release of the stored Ca^{2+} can be observed and monitored by several optical techniques, such as optical flourometric techniques. Elevation of Ca^{2+} ions is highly inhomogeneous because cardiomyocytes consist of several intracellular compartments. Considering this three major cell compartments can be distinguished. One of them is a dyadic or *fuzzy space*. This space is located between the terminal cistern of SR and inner side of the sarcolemma. Second of them is a *submembrane area,* a thin region under the sarcolemma, and the third is a *bulk cytosol* which is the largest part of the intracellular space placed around the contractile proteins.

Calcium reuptake during the calcium efflux

Sequestration of the released Ca^{2+} and stabilization of the diastolic $[Ca^{2+}]$ between contractions is crucial in the regulation of the beat-to-beat Ca^{2+} balance under varying conditions. The majority of the systolic Ca^{2+} increase is reuptaken into the SR by its Ca^{2+} pump (SERCA2a). A much smaller amount of $[Ca^{2+}]_i$ is extruded from the cell, primarily via the forward activity of the NCX. In steady state, during each cycle the released and reuptaken Ca^{2+}, as well as the entered and extruded Ca^{2+} must be equal to avoid excessive Ca^{2+} gain or loss. The NCX, as a passive transporter, is governed by the intra-, and extracellular levels of the Na^{+} and Ca^{2+} ions, and also depends on the actual value of the membrane potential. Based on the direction of the ion transport reverse and forward modes of operation can be distinguished. During reverse mode activity Ca^{2+} influx and an outward current (carried by Na^{+} ions) can be observed, while during forward mode NCX activity Ca^{2+} is extruded and an inward Na^{+} current is generated. Since the operation of the NCX is electrogenic, under certain conditions it may generate significant ionic current in either direction. An ATP-dependent Ca^{2+} transporter, the Ca^{2+} pump of the plasma membrane (PMCA) may also extrude Ca^{2+},

however its contribution to maintain Ca^{2+} balance is much less important, and its role is suggested to be primarily restricted to fine tuning of the diastolic Ca^{2+} level.

Regulation of the calcium homeostasis

Both extrinsic and intrinsic mechanisms contribute to the regulation of the cardiac Ca^{2+} handling. Intrinsic regulation or so called *autoregulation* of the Ca^{2+} means the beat-to-beat control of the released Ca^{2+} on the transmembrane ion fluxes. Autoregulation provides a stable Ca^{2+} level by fine tuning of the SR Ca^{2+} content balancing the influx and efflux. For instance, under experimental conditions, when the SR Ca^{2+} content is low, the CaT is small causing a minimal inhibiting effect on the I_{Ca} and minimal enhancement on the Ca^{2+} efflux through forward NCX. As a consequence, Ca^{2+} influx is more favored than the efflux while the cardiomyocytes getting load with Ca^{2+}.

Role and regulation of the sodium-calcium exchanger

Mammalian Na^{+}/Ca^{2+} exchangers (NCX) are members of a large Ca^{2+}/CA superfamily, whose primary role is to extrude intracellular Ca^{2+}. Three isoforms of NCX exist (NCX1, NCX2, NCX3), but NCX1 is the only isoform expressed in the heart. NCX1 is located in the sarcolemma (primarily in the T-tubules), having 10 transmembrane segments and assembled as a dimmer. Between these segments 5 and 6 is a large cytoplasmic loop playing regulatory role. This loop contains two Ca^{2+} binding domains, CBD1 and CBD2. When extracellular Ca^{2+} is high, Ca^{2+} binds to the CBD domains and increases the channel activity *via* allosteric activation.

The NCX exchanges 3 Na^{+} to 1 Ca^{2+} which implies that the operation of the exchanger is electrogenic. The direction of current corresponds to the mode of operation of NCX: the forward mode operation (Ca^{2+} efflux) generates an inward current, while during the reverse mode (Ca^{2+} influx) function outward current is developed.

The activity of the NCX has a strong dependence on the momentary value of the membrane potential (action potential) and the intracellular as well as extracellular Na^{+} and Ca^{2+} ion levels. Under normal condition the intracellular level of Na^{+}, despite the large magnitude of I_{Na} could be considered relatively constant (8-10 mM). In contrast, the intracellular Ca^{2+} alternates from beat to beat which dynamically governs the actual driving force of the NCX depending on the momentary membrane potential. When the NCX driving force is more positive than the membrane potential, the forward mode is active, while the driving force is more negative than the action potential the reverse mode is facilitated. It is suggested that under normal condition, the reverse mode is restricted in the very early phase

of the action potential, and no longer than 30-50 ms. However the NCX reverse mode is able to contribute to the Ca^{2+} influx which may have a regulatory role in the fuzzy space and/or may contribute to the Ca^{2+} trigger mechanism from the SR. Under pathophysiological condition, such as heart failure, the contribution of the reverse mode is increased providing considerable extra Ca^{2+} influx which may serve as a compensatory mechanism to counterbalance the SR Ca^{2+} loss. The forward mode becomes active when the intracellular Ca^{2+} starts to increase and competes with the SR for the Ca^{2+} extrusion. In rabbit, dog, and human cells the ratio between SERCA and NCX for the Ca^{2+} extrusion is approximately 70:30%. It has important significance during heart failure where the NCX upregulation makes the exchanger nearly 50:50% competitor against SERCA which may contribute to the intracellular Ca^{2+} loss.

Pharmacology of the sodium-calcium exchanger

The selective inhibition of the NCX is a long-standing challenge for the cardiac electrophysiologist. Although many pharmacological agents can inhibit the NCX, the interpretation of these results is difficult by the concomitant effects on other ion transporters or transporter systems. For instance, amiloride analogues were using to study Ca^{2+} homeostasis in cardiac preparations, but results showed that these agents block the voltage gated Ca^{2+} channels. Other drugs such as bepridil or amiodarone are also able to inhibit the NCX, but these molecules are non-selective and their effective concentrations often higher than the concentrations where they exert their primary actions. Benzyloxyphenyl derivate inhibitors like KB-R7943, SEA0400, SN-6 were used successfully in several NCX studies. 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. SEA0400 is a much more potent NCX blocker but both of KB-R7943 and SEA0400 was 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 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 [22].

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. Except for a 20% inhibition of the I_{Kr}, the ORM-10103 did not influence the major ionic currents. More recently a novel compound, ORM-10962 was synthesized exerting considerably lower EC_{50} levels with promising selectivity and antiarrhythmic profile.

Possible clinical implications of exchanger inhibition: antiarrhythmic and positive inotropic effect

Several diseases which increase the level of intracellular Na^+ by reduction of the activity of Na^+/K^+ pump function can lead to marked activation of reverse mode NCX function (such as hypokalaemia), 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. However t*he 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 model. The SEA0400 decreased the incidence, and reduced the development of EADs [39], but 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 paper 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.

The *positive inotropic effect of selective NCX inhibition* is suggested to be based on the development of a new steady-state of the Ca^{2+} handling, where the decreased Ca^{2+} efflux is counterbalanced by the suppressed Ca^{2+} influx via increased CDI of the enhanced released Ca^{2+}. This simple theoretical consideration was seriously challenged by experimental results where NCX inhibition by SEA0400 failed to influence the magnitude of the Ca^{2+} transient in rabbit, guinea pig, and in dog. The earlier studies were made by using SEA0400 having approximately 20% effect on the I_{Ca} however, novel experiments were carried out with the selective inhibitors ORM-10103 and ORM10962, and provided identical results. In contrast, clear inotropic effect was recorded when rats were used as experimental animal [49]. This discrepancy between the results may highlight some specificity of the selective NCX

inhibition as a positive inotropic intervention: such as how does it depend on the shape of the action potential, on the $[Na^+]_i$ and $[Ca^{2+}]_i$ levels, and on the actual balance between NCX working modes.

AIMS OF THE STUDY

Previous results suggest that the selective inhibition of the NCX could be either cause Ca^{2+} gain or loss depending on its actual transport balance: reverse mode facilitation promotes Ca^{2+} loss while forward mode induction may cause intracellular Ca^{2+} gain. This observation may have crucial importance since suppression of different dominant transport modes could be attributable to distinct possible clinical outcomes: inhibition of reverse mode could be antiarrhythmic while reduction of forward mode may cause positive inotropic effect. In order to address this issue novel completely selective NCX inhibitors are required without any effect on the Ca^{2+} current. Therefore in this thesis we would like to investigate and clarify the effectiveness of a novel compound GYKB-6635 on the NCX current. and to investigate and explain how does the shifts of the actual transport kinetics of the NCX influence the possible negative and positive inotropic effect of a novel NCX inhibitor ORM-10962, under various experimental conditions (low Na+o, high Ca2+i, low K+o).

MATERIALS AND METHODS

Animals

Cardiac ventricular myocytes were obtained from hearts of adult mongrel dogs of both sexes (10-16 kg). Wistar rats 200-250 g obtained from a licensed supplier were used for the study. Following anticoagulation by sodium-heparin, animals were sedated xylazine (1 mg/kg, i.v.), then anaesthetized (thiopental, 30 mg/kg i.v.). Each heart was rapidly removed through a right lateral thoracotomy and rinsed in Tyrode's solution (NaCl 144, NaH_2PO_4 0.4, KCl 4.0, $CaCl_2$ 1.8, $MgSO_4$ 0.53, glucose 5.5 and HEPES 5.0, at pH of 7.4) in the case of dog and in modified Locke's solution (containing in mM: NaCl 120, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1, $NaHCO_3$ 22, glucose 11) in the case of rats. The pH of the Locke's solution was set between 7.35 and 7.4 when saturated with the mixture of 95% O_2 and 5% CO_2 at 37 ºC.

Measurement of Na^+/Ca^{2+} exchanger (NCX) current

For the measurement of the Na^+/Ca^{2+} exchanger current (I_{NCX}) different protocols were used. The conventional "ramp" protocol [50] was applied to estimate the effectiveness of GYKB-6635. Accordingly, the NCX current is defined as Ni^{2+}-sensitive current and measured in a

special K^+-free solution (composition in mM: NaCl 135, CsCl 10, $CaCl_2$ 1, $MgCl_2$ 1, $BaCl_2$ 0.2, NaH_2PO_4 0.33, TEACl 10, HEPES 10, glucose 10 and ouabain 20 μM, nisoldipine 1 μM, and lidocaine 50 μM, at pH 7.4) after the formation of the whole cell configuration in HEPES-buffered Tyrode's solution. The pipette solution used for recording I_{NCX} is contained in (mM): CsOH 140, aspartic acid 75, TEACl 20, MgATP 5, HEPES 10, NaCl 20, EGTA 20 and $CaCl_2$ 10, pH was adjusted to 7.2 with CsOH. The [Ca^{2+}] in the pipette was 160 nM by applying an appropriate mixture of $CaCl_2$ and EGTA as calculated by the WinMaxC software [51]. After recording of control current, GYKB-6635 was applied, and finally 10 mM $NiCl_2$ was administrated to estimate the total NCX current. Therefore the inhibited fraction was calculated as a subtracted current: the trace recorded in the presence of 10 mM $NiCl_2$ was subtracted from that measured in the absence of $NiCl_2$. The current-voltage (I-V) relationship of NCX was measured through the use of ramp pulses at 20 s intervals. The ramp pulse initially led to depolarization from the holding potential of -40 mV to 60 mV with a rate of 100 mV/s, then the cell was hyperpolarized to -100 mV, and depolarized back to the holding potential. The descending limb of the ramp was utilized to plot the I-V curve.

In the other sets of experiments NCX current was estimated in the presence of intact Ca^{2+} handling. The cells were paced at 1 Hz applying 0 or + 30 mV voltage pulses through the patch pipette to activate I_{CaL} and NCX where the microelectrode solution did not contain nor EGTA nor Ca^{2+}. Further detailed descriptions of the experiments can be found at the Results section.

Measurement of L-type calcium current

In the experiments where Ca^{2+} handling was necessary to be suppressed the L-type calcium current (I_{CaL}) was recorded in HEPES-buffered Tyrode's solution supplemented with 3mM 4-aminopyridine, to block all potassium currents. A special solution was used to fill the micropipettes (composition in mM: KOH 40, KCl 110, TEACl 20, MgATP 5, BAPTA 10, HEPES 10 and GTP 0.25, pH was adjusted to 7.2 by KOH). I_{CaL} current was evoked by 400 ms long depolarizing voltage pulses 0 mV. The holding potential was -80 mV. A short prepulse to -40 mV served to inactivate Na^+ current. The amplitude of I_{CaL} was defined as the difference between the peak inward current at the beginning of the pulse and the current at the end of the pulse.

Recording of the cell shortening

The rat ventricular isolated cells were field stimulated with 1Hz through a pair of platinum electrodes while were continuously perfused with normal and modified Tyrode's solutions in the following sequence: (*i*) Normal (4.5 mM KCl) Tyrode's solution for 10-15 min; (*ii*) hypokalemic conditions were induced by perfusing with a modified Tyrode' solution (2.7 mM KCl) *(iii)* modified Tyrode's solution with a presence of 1 μM ORM10962. Perfusion was taken for 10 minutes. Throughout the entire procedure, solutions temperature of the solutions were maintained at 37 °C. Cell shortenings were recorded at every 2nd minute as PC video files and analyzed off-line using a MatLab-based CellContract 1.0 software.

Recording CaT from field stimulated canine ventricular cells

Myocytes were loaded with Fluo-4 AM (5 μM) for 15 min at room temperature in Tyrode's solution. One drop of the cells was placed in a cell chamber (RC47FSLP, Warner Instruments, Hamden, CT, USA) and were field stimulated at 1 Hz with a stimulator (PW-01, Experimetria Ltd. Hungary). The measurement was performed on an inverted microscope (Olympus IX 71; Olympus, Tokyo, Japan). The fluorescent dye was excited at 480 nm and emitted at 535 nm. Ca^{2+} signals were recorded by a photon counting photomultiplier module (Hamamatsu, model H7828; Hamamatsu Photonics Deutschland GmbH, Herrsching am Ammersee, Germany) and were digitized and sampled at 1 kHz by using Digidata 1440A interface (Axon Instruments). The data acquisition and analysis were performed by pClamp 10.0 (Molecular Devices, USA). The transient amplitudes were calculated as a difference of the peak and diastolic fluorescent values. Background fluorescence levels were used to correct raw fluorescence data. The Ca^{2+} transients were normalized to the diastolic fluorescent level.

Data analysis and statistics

All data are expressed as means ± SEM. Statistical analysis was performed with Student's t-test for paired data. The results were considered statistically significant when the p value was < 0.05.

RESULTS

Effects of GYKB-6635 on the NCX current

The GYKB-6635 effectively inhibited the NCX current in the micromolar range (**Fig.3**) without any effect on the I_{Ca} or on the major K^+-currents (I_{Kr}, I_{Ks}, I_{K1}). This study [54] further showed that GYKB-6635 was effective against DAD-related arrhythmias on guinea-pig Langendorff-perfused hearts. The improved effectiveness and selectivity of GYKB-6635

obviously highlights from the previous compounds: KB-R7943, SEA0400, and ORM-10103 all have inhibitory effects on I_{CaL} or I_{Kr} which makes the data interpretation difficult. In contrast, GYKB-6635 could be a novel potential experimental as well as therapeutical tool to investigate the NCX function under physiological or in pathological condition.

Effects of ORM-10962 on I_{Ca}

Our main finding is that ORM-10962 has no direct suppressive effect on L-type Ca^{2+} current, however it influences the I_{Ca} in the presence of intact Ca^{2+} handling. This is based on the following findings: (1) The magnitude of I_{Ca} traces did not change in the presence and absence of ORM-10962 when Ca^{2+} cycling was substantially buffered by BAPTA. (2) ORM-10962 had no influence on I_{Ca} when the Ca^{2+} content of the SR was depleted by caffeine. (3) The inhibitory effect of ORM-10962 on I_{Ca} amplitude restored as SR became refilled allowing for gradually increasing Ca^{2+} release. (4) ORM-10962 markedly suppressed I_{Ca} under conditions of normal Ca^{2+} cycling. These observations are essential for two major reasons. Firstly, indirect reduction in Ca^{2+} influx may be involved in compensation for results of forward mode NCX inhibition and perhaps therefore limit the expected positive inotropy. Secondly, the secondary reduction in Ica is a strong evidence of increasing of $[Ca^{2+}]_i$.

Marginal positive inotropy under baseline conditions

Previous study from our laboratory demonstrated that application of 1 μM ORM-10962 has approximately 90% inhibitory effect of NCX, measured by conventional ramp protocol. These results justify the 1 μM chosen concentration in our experiments. Since the relative contribution of the reverse mode NCX activity is believed to be moderate under baseline conditions, consequences of forward mode inhibition should be more pronounced. Therefore an approximately 90% forward mode NCX blockade should produce a significant Ca^{2+} accumulation in the SR with a following increase in Ca^{2+} release and positive inotropy Surprisingly, under normal conditions the application of ORM-10962 caused a marginal positive inotropic action. The underlying mechanisms may consist of two main categories: (1) possibly ORM-10962 causes an asymmetrical blockade on NCX under normal Ca^{2+} handling and (2) reverse and forward mode inhibition may have asymmetrical consequences in the Ca^{2+} handling.

Inotropic effect of NCX inhibition depends on the relative contribution of reverse NCX activity

A significant negative inotropic action of NCX blockade was described in our previous study, when reverse mode was facilitated by low NaCl containing Tyrode solution. According to

these previous results in this study we report that application of ORM-10962 reduced the SR Ca^{2+} content following an earlier enhancement of the reverse mode of NCX activity. In our earlier work, similar observations were made with a previous NCX inhibitor ORM-10103. Where ORM-10103 completely reversed or prevented the elevation of $[Ca^{2+}]_i$, when reverse mode was facilitated by veratrine or ATXII. For comparison, our previous and present study demonstrated that the positive inotropic effect of the forward mode blockade (when reverse mode was suppressed) was considerably weaker than was expected during ~ 90 % inhibition of NCX.

Possibility of an asymmetrical NCX blockade

Even though some evidences demonstrating that mode selective NCX inhibition may occur under certain experimental conditions, theoretical considerations do not support the possibility of transport mode dependent inhibition.

Previous papers demonstrated that KB-R7943 and SEA0400 preferentially block the reverse mode NCX. In our previous studies, ORM-10103 or ORM-10962 exerted balanced inhibitory effect between reverse and forward mode, therefore we assume that the possibility of asymmetrical inhibition of the reverse and forward mode as a basis for the limited positive inotropic effects can be ruled out in our experiments.

The forward mode NCX blockade may be alleviated by another potential mechanism and thus the positive inotropic effect is the possible modulatory effect of Ca^{2+} on the NCX inhibition. Such a phenomenon has been implicated in the case of KB-R7943. When SEA0400 was applied to block NCX in a previous study, some evidence was found which supporting our theory.

Asymmetrical consequences of NCX inhibition in Ca^{2+} handling

Asymmetrical consequence means that the ORM-10962 pharmacologically equally inhibits both mode of function, however the unequal ratio of reverse and forward mode during a cycle may cause asymmetrical effect on Ca^{2+} handling, under experimental conditions favouring the forward mode NCX activity, the observed positive inotropy highlights the importance of adaptive changes in the balance of the Ca^{2+} influx and efflux. These mechanisms may consist of three major submechanisms in the Ca^{2+} handling:

(1) Adaptive changes in Ca^{2+} influx via I_{Ca} due to the increased submembrane $[Ca^{2+}]_i$.(2) The elevated submembrane $[Ca^{2+}]_i$ activates an alternative Ca^{2+} removal mechanism (e.g. the sarcolemmal Ca^{2+} ATP-ase). In this case the contribution of reverse mode NCX activity to normal Ca^{2+} handling is strongly underestimated or the importance of forward mode NCX

activity in Ca^{2+} removal is overestimated. (3) Preserved inducibility of forward NCX activity by high $[Ca^{2+}]_i$ following NCX blockade.

NCX inhibition reverts the incidence of arrhythmias and Ca^{2+} overload induced cell shortening in hypokalaemia

The increase of reverse NCX mediated Ca^{2+} influx by elevation of intracellular Na^+ level not only promotes the outward NCX ratio during a Ca^{2+} handling cycle, but also augments the intracellular Ca^{2+} levels resulting in an increased driving force for forward NCX. Our previous results in this study may suggest that in this condition the inward NCX inhibition was attenuated and the effect of reverse NCX blockade will primarily prevail under this circumstance and net Ca^{2+} loss is expected. As was hypothesized, in the presence of hypokalaemic solution the Ca^{2+} transient was increased presumably after suppression of the Na^+/K^+ pump and consequential Na^+_i gain which activates Ca^{2+} influx through reverse NCX on rat isolated myocytes. This improved Ca^{2+} level was decreased after NCX inhibition which could be attributable to the outward exchanger inhibition. These results may further support the hypothesis of asymmetrical functional consequences of NCX blockade. As was expected, the adverse effects of elevated intracellular Ca^{2+} such as Ca^{2+} waves or extra beats were also suppressed. It is important to mention here that a substantial species-dependent effect was discovered between rat and dog myocytes regarding NCX inhibition mediated positive inotropy: in our previous studies on rat we found marked increase in Ca^{2+} transient and cell shortening/contraction after NCX inhibition, and we failed to reproduce this effect on dog and rabbit myocytes under normal condition. Positive inotropic effect of selective NCX inhibition was observable only after augmentation of Ca handling (independently from reverse NCX activation) which may suggest some important functional differences of forward NCX function between species. The different degree of forward NCX on Ca^{2+} extrusion between species may contribute to this discrepancy. However such interspecies difference was not observed after reverse NCX stimulation: hypokalaemia increased the intracellular Ca^{2+} load which was suppressed after ORM application, in parallel with our previous results carried out on dog myocytes. These results may implicate uniformity of reverse NCX blockade among different species, at the same time may indicate different capacity of forward NCX for Ca^{2+} extrusion between rats and dogs.

These experiments may indicate that when the NCX balance is shifted toward facilitated reverse mode function, the NCX inhibition resulted in uniformly a decrease of the Ca^{2+} level, suggesting asymmetrical, reverse NCX-dominant inhibition. However under normal

condition, positive inotropic effect was described only in rats and marginal effect was observed in the case of guinea-pig, rabbit, and dogs. These animals have considerable difference in baseline heart rate, in the action potential shape and in the underlying ionic currents which may contribute to this discrepancy. However, regarding the function of the forward NCX it is important to note that "functional capacity" of NCX may be increasing from rat to dog, providing an important reserve for Ca^{2+} extrusion. The underlying mechanism of this phenomenon is not clear, it could be related to the autoregulatory mechanism, and/or the persevered inducibility of the NCX in the presence of high Ca^{2+}_i.

SUMMARY

The major goal of the cardiac Ca^{2+} handling is to control the actual magnitude and kinetics of the cell contractions and therefore contribute to the regulation of the cardiac output. The Na^+/Ca^{2+} exchanger has a crucial role in the beat-to-beat Ca^{2+} balance by extruding a relatively small fraction of the released Ca^{2+}. Since the exchanger works Ca^{2+} influx, as well as Ca^{2+} efflux mode in the same heart cycle, its inhibition theoretically could lead to Ca^{2+} loss or Ca^{2+} gain. The functional consequence of these effects could be manifested in negative inotropy which may have antiarrhythmic effects during Ca^{2+} overload, or could cause positive inotropy which is desired in heart failure. However the exact electrophysiological mechanism which determines these two outcomes is not clarified because of the lack of selective inhibitors. In this thesis we would like to address these issues by using novel, selective NCX compounds. The main results can be summarized as follows:

1) The novel NCX inhibitor GYKB-6635 effectively suppressed both mode of the NCX current measured by conventional ramp protocol. Our study showed the GYKB-6635 did not influence the kinetics of the L-type Ca^{2+} current and major K^+-currents therefore it could be considered a promising experimental tool for future NCX research.

2) The selective NCX inhibition is able to cause both positive and negative inotropy by ORM-10962 in the cardiac cells, depending on the experimental condition. When the reverse mode is facilitated Ca^{2+} loss, in the case of forward mode enhancement Ca^{2+} gain occurs. The major underlying mechanism is the actual Ca^{2+} level of the sarcoplasmic reticulum which strongly depends on the function of the NCX.

3) The selective NCX inhibition by ORM-10962 reverts the hypokalaemia induced positive inotropy: the low $[K^+]_o$ increases the intracellular Na^+ level of the cells which shifts the actual reversal potential of the NCX facilitating reverse mode. The selective NCX inhibition may

inhibit preferentially the reverse mode of the NCX under this setting which decreases the intracellular Ca^{2+} and cell shortening.