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

PhD Thesis

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Hungary

2018

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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.

ABSTRACTS

I. Prorok J, **Oravecz K**, Gazdag P, Frey Zs, Vígh D, Nagy N, Acsai K, Tóth A, Jost N, Papp JGy, Varró A
Az NCX szelektív gátlása csökkenti a hypokalaemia indukált emelkedett intracelluláris Ca^{2+} szintet és a kamrai aritmiák kialakulását (Selective inhibition of the NCX attenuates the hypokalaemia-induced elevated intracellular Ca^{2+} load and decreases the incidence of ventricular arrhythmias)
Cardiologica Hungarica 47:(Suppl.C) pp. C42-C43. (2017)

II. Prorok J, **Oravecz K**, Gazdag P, Kui P, Takács H, Polyák A, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Nagy N, Farkas AS, Tóth A, Papp JGy, Varró A, Acsai K
Pharmacological inhibition of the Na^{+}/Ca^{2+} exchanger attenuates the hypokalaemia-induced elevated cellular Ca^{2+} load and decreases the risk of arrhythmia
Current Research: Cardiology – Experimental Clinical 3:(3) P. 112. (2016)

III. Acsai K, **Oravecz K**, Kormos A, Marton Z, Papp JG, Varro A
Shift in Na^{+}/Ca^{2+} exchange balance modulates the inotropic consequences of the NCX inhibition
Acta Physiolgica 211:(697) pp. 93-94. (2014)

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

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.

1. 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 [1], Nevertheless selective inhibitor has not been available so far. However the recently synthesized, selective compounds may provide novel perspectives in the future drug therapy.

1.1. 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 [2].

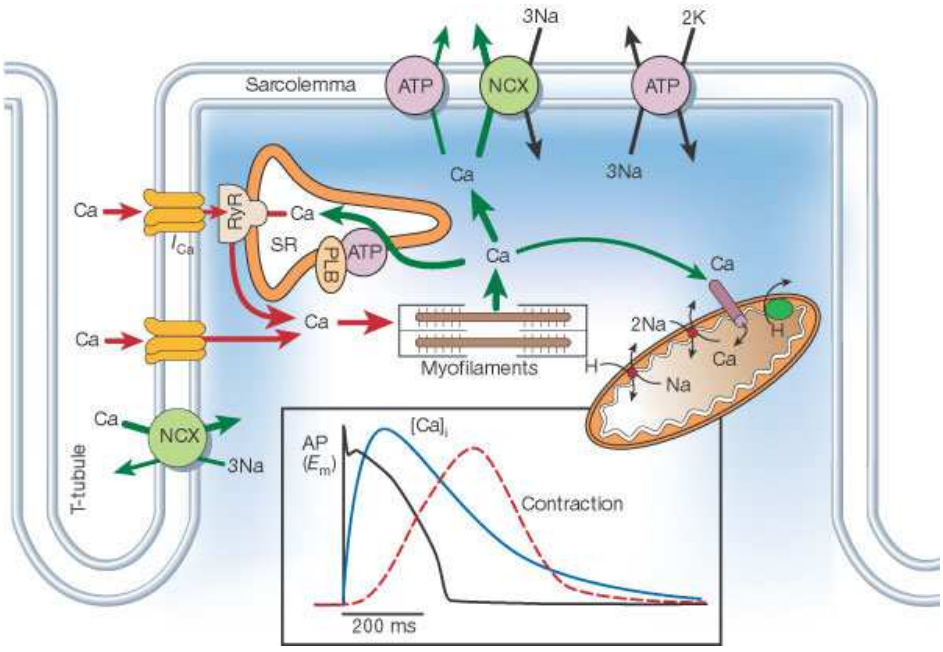

Figure 1.: Intracellular Ca^{2+} homeostasis of the cardiac cell. *The upper panel shows the main routes of the Ca^{2+} movements of the cell, the inset shows the time course of an action potential, Ca^{2+} transient and contraction. NCX: Na^+ /Ca^{2+} exchange; SR: sarcoplasmic reticulum, PLB, phospholamban; SR, sarcoplasmic reticulum.* [2].

1.2. 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 [3] as well as Na^+ channels [4-6] 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 [5]. 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) [2].

1.3 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 [2, 7].

The amount of Ca^{2+} near to the myofilaments is determined by the sum of the released Ca^{2+} from the SR and the amount of Ca^{2+} which is diffused from the fuzzy space. Ca^{2+} in the dyadic space reaches a considerably larger magnitude and declines much faster compared to that in the bulk cytosol during the CaT therefore the ion channels and transporters placed in the membrane are faced to remarkably larger beat to beat Ca^{2+} fluctuations than what can be measured in the cytosol by the general fluorometric techniques [8].

1.4. 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 [2, 7].

1.5. 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+}. Elevated SR Ca^{2+} content permits larger CaT after some beats. A higher CaT restrains influx and promotes efflux until sarcolemmal Ca^{2+} fluxes are balanced. This mechanism is an intrinsic property of the cardiac cells Ca^{2+} homeostasis and does not require any external regulation such as β-adrenergic activation [9].

Neurohormonal system has a main role and multiple effects on the extrinsic regulation. This system modifies the activity of the heart to arrange the body's requirement in varied physiological and pathological conditions. Signaling pathways of cAMP and protein-kinase-A (PKA) and bindings of their metabolites on beta-adrenergic receptors have a main role to modify the regulation of the heart activity. Due to the phosphorilation of Ca^{2+} channels, RyR, $SERCA_{2a}$, troponin and phospholamban, an increased Ca^{2+} influx and reuptake are caused therefore this leads to a faster reuptake and stronger contraction. Other signaling pathways such as activation of protein-kinase-C also have an important role on several ionic currents [10].

1.6. 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), [11, 12] having 10 transmembrane segments and assembled as a dimmer [13]. 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 [14]. 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.

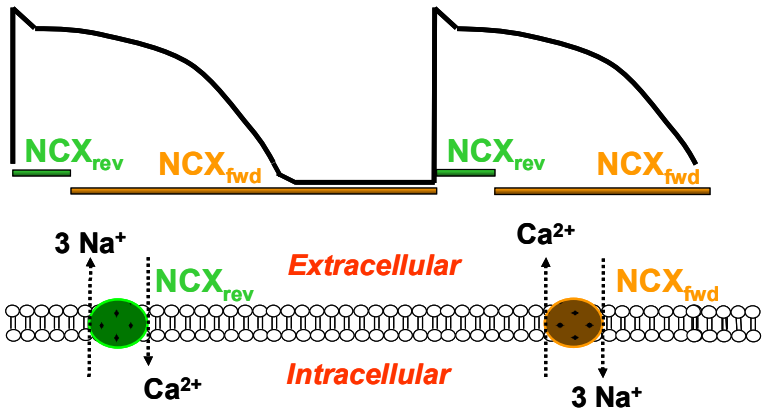

Figure 2.: Schematic presentation of the two modes of NCX current under the action potential. *The reverse mode of the NCX (labeling with green) (outward current) is only presented in the early phase of the AP, when NCX extrudes 3 Na^+ from the cell and moving Ca^{2+} into the cell making an outward current. The forward mode NCX (inward current) is active during most of the AP (labeled with orange.) and the Ca^{2+} extrusion is coupled with three Na^+ ion entries to the cell.*

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 [10]. However the NCX reverse mode is able to contribute to the Ca^{2+} influx which may have a regulatory role in the fuzzy space [12] and/or may contribute to the Ca^{2+} trigger mechanism from the SR [15]. 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% [2]. 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.

1.7. 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 [1, 16]. 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 [16]. Benzyloxyphenyl derivate inhibitors like KB-R7943, SEA0400, SN-6 were used successfully in several NCX studies [1]. 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 [17]. 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, [18, 19], 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 [20], 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 [21]. 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 [23] 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 [24].

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

Pharmacological modulation of the NCX has been emerged as one of the most promising novel therapeutic target in the past decade. The possible outcome of the inhibition of NCX is related to the NCX operational modes: the reverse mode gains the Ca^{2+} level of the cell which suppression could be beneficial during Ca^{2+} overload while the forward mode extrudes Ca^{2+} which reduction may increase the Ca^{2+} content of the cell and therefore causes positive inotropy [24, 25].

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 [26]. 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 [1]. NCX inhibitors have shown antiarrhythmic effects in hearth rhythm disturbances evoked by ischemia/reperfusion injury *in vivo* [27], in Langendorff

perfused hearts [28-33], and in pharmacologically simulated ischemia/reperfusion models [34-38]. The SEA0400 decreased the incidence [38], and reduced the development of EADs [39], but it failed to suppress the aconitine induced arrhythmias [40]. In Langendorff-perfused rat hearts SEA0400 even enhanced the arrhythmia incidence and duration [41]. 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 [42, 43], while in another paper it effectively reduced the amplitudes of EADs, without influencing APD [38]. In contrast, Milberg et al reported considerable APD shortening effect of SEA0400, furthermore, sotalol or veratridine induced TdPs were also suppressed [39, 44]. Recently Jost et al. [23] claimed that ORM-10103, a novel NCX inhibitor with improved selectivity decreased pharmacologically induced DADs and EADs, confirming previous results performed with SEA0400 [38].

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 [43], guinea pig [45], and in dog [46]. The earlier studies were made by using SEA0400 having approximately 20% effect on the I_{Ca} [47] however, novel experiments were carried out with the selective inhibitors ORM-10103 and ORM10962, and provided identical results [24, 48]. 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.

1.9. 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 [24, 25]: 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 Ca^{2+}_{i}, low K^{+}_{o}).

2. MATERIALS AND METHODS

2.1. Ethical consideration

All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No 85-23, revised 1996) and conformed to Directive 2010/63/EU of the European Parliament. The protocols were approved by the review board of the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (XIII./1211/2012). The ARRIVE guidelines were adhered to during the study (NC3Rs Reporting Guidelines Working Group, 2010).

2.2. 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.

2.3. Cell preparation

2.3.1. Isolation of canine ventricular myocytes

Segment perfusion method was used to obtain ventricular cells. Segment of the left ventricular wall containing an arterial branch was cannulated and perfused using modified Langendorff apparatus at a pressure of 60 cmH_2O, with solutions in the following sequence: (*i*) isolation solution supplemented with $CaCl_2$ (1 mM) and sodium-heparin (0.5 ml) for 5-10 min to wash out the blood from the tissue; (*ii*) Ca^{2+} free isolation solution for 10 min; (*iii*) isolation solution (150 mL) supplemented with collagenase (type II, 250U/ml; Worthington) and 33 μM $CaCl_2$ for 15 min. Protease (type XIV, 0.04 mg·mL−1; Sigma Chemical) was added at the 15th minute of the third section of isolation and further 15-20 min of digestion was allowed. Portions of the left ventricular wall from the midmyocardial part were cut into

small pieces in isolation solution supplemented with $CaCl_2$ (1 mM) for 15 min in 37°C. These tissue samples were then gently agitated in a small beaker to dislodge single myocytes from the extracellular matrix. Throughout the entire isolation procedure, solutions were gassed with 100% O_2, while their temperature was maintained at 38 °C. Myocytes were allowed to settle to the bottom of the beaker for 10 min, and then half of the supernatant was replaced with fresh solution. This procedure was repeated three times. Myocytes placed in isolation solution supplemented with $CaCl_2$ (1 mM) were maintained at room temperature before the experiment.

2.3.2. Isolation of rat ventricular myocytes

Modified Langendorff apparatus was used to gain ventricular cells at a pressure of 60 cmH_2O, with solutions in the following sequence: (*i*) isolation solution supplemented with $CaCl_2$ (1 mM) and sodium-heparin (0.5 ml) for 5-10 min to wash out the blood from the tissue; (*ii*) Ca^{2+} free isolation solution for 12 min; (*iii*) isolation solution (100 ml) supplemented with collagenase (type I, 25mg/100ml; Worthington) and hyaluronidase (25mg/100ml) and 180 μM $CaCl_2$ for 8-10 min. Portions of the ventricles were cut into small pieces and then filtered through in KB solution, containing in (mM): Glutamic acid 70, Taurine 15, KCl 30, KH_2PO_4 10, HEPES 10, $MgCl_2$ 0.5, Glucose 11, EGTA 0.5; pH=7.3 with KOH. Throughout the entire isolation procedure, solutions were gassed with 95% O_2 and 5% CO_2, while their temperature was maintained at 38 °C. Myocytes were allowed to settle to the bottom of the beaker for 10 min, and then half of the supernatant was replaced with fresh solution. This procedure was repeated two times. Myocytes were maintained at room temperature before the experiment.

2.4. Voltage-clamp measurements

One drop of cell suspension was placed in a transparent recording chamber mounted on the stage of an inverted microscope (Olympus IX51, Olympus, Tokyo, Japan), and individual myocytes were allowed to settle and adhere to the chamber bottom for at least 5-10 min before superfusion was initiated and maintained by gravity. Only rod-shaped cells with clear striations were used. Normal Tyrode solution was used as the normal superfusate. Micropipettes were fabricated from borosilicate glass capillaries (Science Products GmbH, Hofheim, Germany), using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA), and had a resistance of 1.5-2.5 MOhm when filled with pipette solution. The membrane currents were recorded with Axopatch-200B amplifiers (Molecular Devices,

Sunnyvale, CA, USA) by applying the whole-cell configuration of the patch-clamp technique. The membrane currents were digitized with 250 kHz analogue to digital converters (Digidata 1440A, Molecular Devices, Sunnyvale, CA, USA) under software control (pClamp 10, Molecular Devices, Sunnyvale, CA, USA). Experiments were carried out at 37 °C.

2.4.1. 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.

2.4.2. 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.

2.5. 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.

2.6. 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.

2.7. Chemicals

With the exception of ORM-10962 (Orion Pharma, Espoo, Finland) all chemicals were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). ORM-10962 was synthesised by the Orion Pharma, Espoo, Finland. The GYKB-6635 was synthesized by the

Department of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary. ORM-10962 and GYKB-6635 were dissolved in DMSO to obtain a 10 mM stock solution. These stock solutions were diluted to reach the desired final concentration (DMSO concentration not exceeding 0.1 %) in the bath. The stock solutions were stored at 4 °C. All solutions used were made freshly prior to the experiment.

2.8. 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.

3. RESULTS

3.1. Investigation of the NCX inhibition under normal experimental circumstances

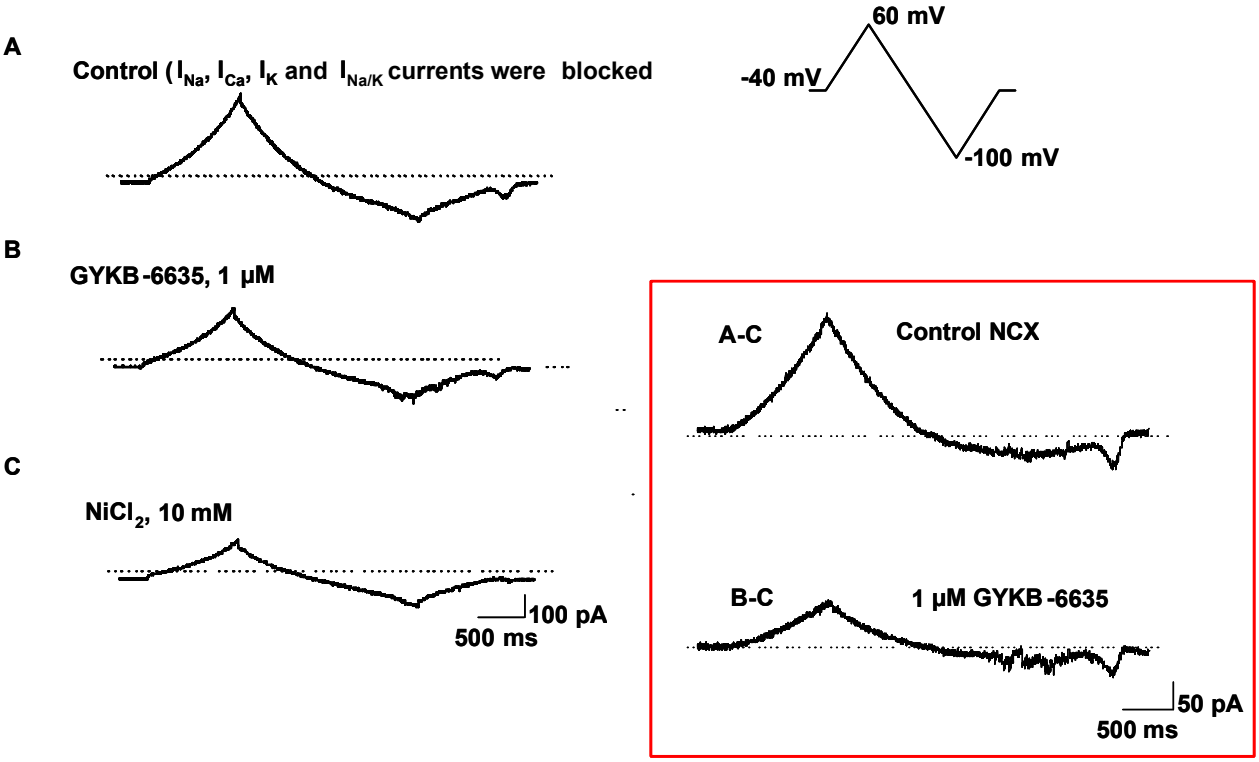

Figure 3.: Determination of sodium–calcium exchanger (NCX) current in canine ventricular myocytes. *The protocol was adapted from Hobai et al. (1997)* [50]. *(A) Recording obtained with the voltage protocol shown in the inset in control conditions (current trace after blockade of Na^+, Ca^{2+}, K^+, and Na^+/K^+ pump currents). (B) The current trace after superfusion with 1 µM GYKB-6635. (C) The current trace at the end of the measurements after the application of 10 mM $NiCl_2$. On the right, the control NCX current is shown, which is obtained by subtracting trace (C) from trace (B). The NCX current in the presence of 1 µM GYKB-6635 is obtained by subtracting trace (B) from trace (C). Note the difference in the intensity-time calibration in the left and right panels.*

The NCX current was measured by using the conventional "ramp" protocol which was described in detail earlier [23]. After control measurements 1 µM GYKB-6635 was applied and finally, the total NCX current was calculated as Ni^{2+} sensitive current after application of 10 mM $NiCl_2$ (**Figure 3**). Ramp pulse was applied from a holding potential of -40 mV to 60 mV (1 s duration) and repolarized to -140 mV (2 sec duration). 1 µM GYKB-6635 reduced by 57% the reverse NCX current (from 52.5 ± 18.5 pA in controls to 22.4 ± 11.9 pA after drug administration, $n = 4$, $p < 0.05$), while the forward current was reduced by 58% (from –52.7 ± 21 pA in controls to –21.8 ± 12.3 pA after drug administration, $n = 4$, $p < 0.05$).

3.2. Inotropic effects of NCX inhibition under normal condition

Inotropic effect of selective NCX inhibition was investigated on cell shortening and Ca^{2+} transient under normal condition (140 mM $[Na^+]_o$). Cardiac myocytes were field stimulated at 1 Hz and 1 µM ORM-10962 was applied after steady-state control recordings. Representative traces are shown at **Figure 4.A-B**. Amplitude of cell shortening and Ca^{2+} transient are significantly increased after application of 1 µM ORM-10962. The means of the observed changes is depicted by bar graphs (**Figure 4C**; transient: 4.22±1.7%; cell shortening: 17.32±2.84%, n=6 respectively, $p<0.05$). These results were practically identical with previous data from our laboratory with ORM-1096, 10103 and SEA-0400 [24, 46].

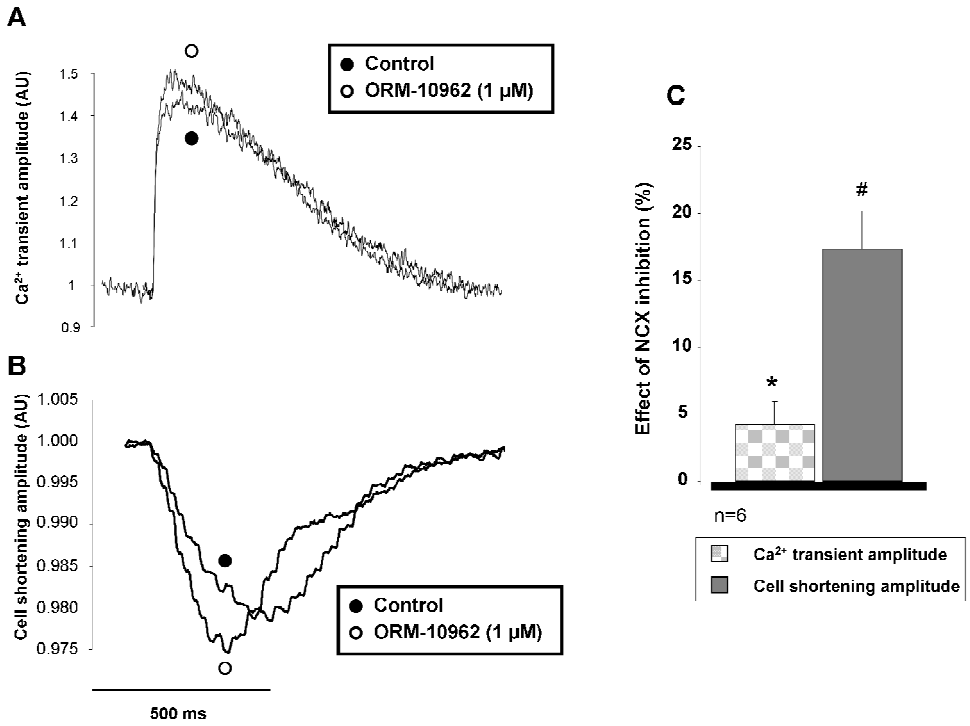

Figure 4.: Evaluation of positive inotropy of 1 µM ORM-10962 on isolated canine ventricular cells under normal conditions. *Panels A and B show superimposed representative traces of Ca^{2+} transients (A) and CS (B) under control conditions (filled symbols) and after exposure to 1 µM ORM-10962 (open symbols). The cells were paced using field stimulation at 1 Hz. Average results obtained from 6 myocytes are presented in panel C. Columns and bars represent mean ± SEM values, asterisks denote significant changes from control.*

3.3. Effects of NCX inhibition on SR Ca^{2+} content

Ca^{2+} content of the SR is important to determine the actual SR Ca^{2+} release and influences the force of cell contraction. The change in SR Ca^{2+} content might reflect the net effect of NCX

inhibition (when other factors do not change) in order to the balance between a sarcolemmal Ca^{2+} influx and efflux. For this reason, total SR Ca^{2+} content under different experimental circumstances was estimated by integrating the inward NCX current. Holding potential was obtained at -80 mV in response to 10 mM caffeine [52]. Rapid exposure of caffeine releases the total Ca^{2+} stored in the SR which is extruded by the forward NCX. The integral of the inward current reflects the total SR Ca^{2+} content. Illustrative examples of the inward NCX current evoked by caffeine –induced Ca^{2+} release before and after NCX inhibition, and the collateral changes in the SR Ca^{2+} content under various conditions are shown in (**Figure 5.C and D**). Reduced $[Na^+]_o$ concentration to 70 mM (**Figure 5.A and C**) caused a suppression of the transmembrane Na^+ gradient, which caused an elevation at the calcium amount stored in the SR (not shown). Elevation was significantly declined by the application of ORM-10962 (from 2.19±0.2 to 1.53±0.17 fmol, n=7, $p<0.05$).

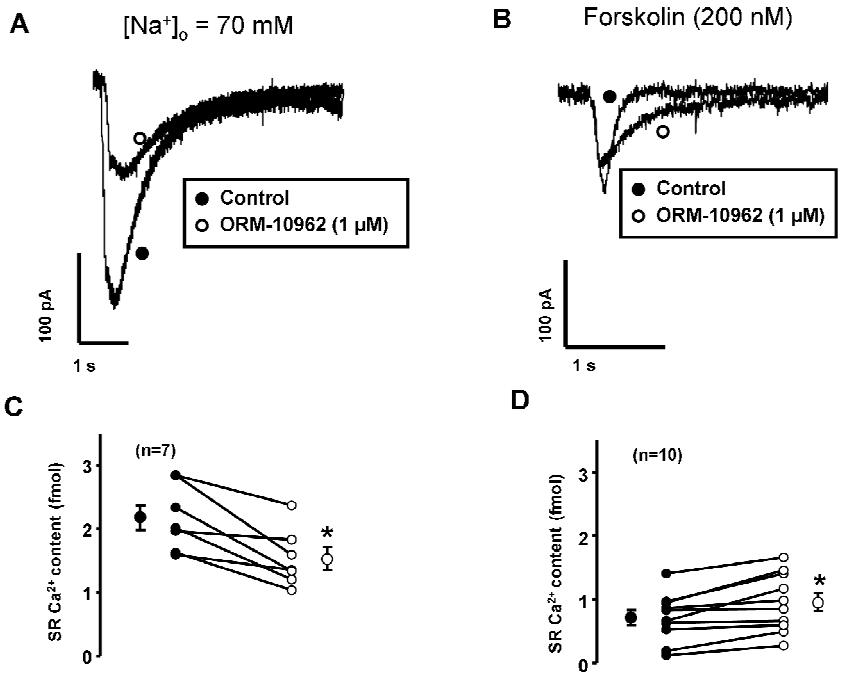

Figure 5. : Effects of 1 µM ORM-10962 on the Ca^{2+} content of the SR. Upper row: *Superimposed inward NCX current traces evoked by a 10 mM caffeine pulse at low $[Na^+]_o$ (A), and in the presence of 200 nM forskolin (B). SR Ca^{2+} content was calculated from the integral of I_{NCX}. The corresponding individual and average data, obtained in the absence and presence of ORM-10962 (filled and open symbols, respectively) are presented in the bottom row (C, D). Symbols and bars represent mean ± SEM values, asterisks denote significant changes induced by ORM-10962 in 7 and 10 preparations, respectively.*

In comparison, NCX inhibition by ORM-10962 significantly elevated the SR Ca^{2+} content, when forward mode NCX activity was favored by application of 200 nM forskolin in the presence of low voltage stimulation pulses and low pipette $[Na^{+}]_i$ concentration (from 0.72±0.12 to 0.95±0.14 fmol, n=10, $p<0.05$; **Figure 5B-D**). The above mentioned results claimed that the observed reduction or enhancement in cell shortening correlates with the Ca^{2+} content of SR. These results suggest that SR Ca^{2+} content could be a key parameter in determining the inotropic effect of NCX inhibition.

3.4. ORM-10962 inhibits the reverse NCX mediated positive inotropy

To investigate further the above mentioned observations, transmembrane currents were recorded using the whole cell patch clamp technique parallel with cell shortening.

At these experiments cells were stimulated at 1 Hz with pulses clamped to +30 mV from the holding potential of –70mV. Positive stimulatory potential was used and the Na^{+} content of the external solution was reduced to 70 mM in order to facilitate Ca^{2+} influx via reverse mode NCX activity. Representative traces of membrane currents recorded under these conditions are shown in **Figure 6A**. After application of ORM-10962 outward current measured at the end of the +30 mV pulse. Outward current is significantly decreased (from 150±29 to 82±18 pA, $p<0.05$, n=8), demonstrating an inhibition of the outward NCX current generated by the reverse mode NCX (**Figure 6C**). This marked reduction in outward NCX current was accompanied by a considerable reduction in CS (from 7.7±1.3 to 4.9±1.2 %, $p<0.05$, n=7), indicating the elimination of positive inotropy mediated by the previously activated reverse mode NCX (**Figure 6 B-D**).

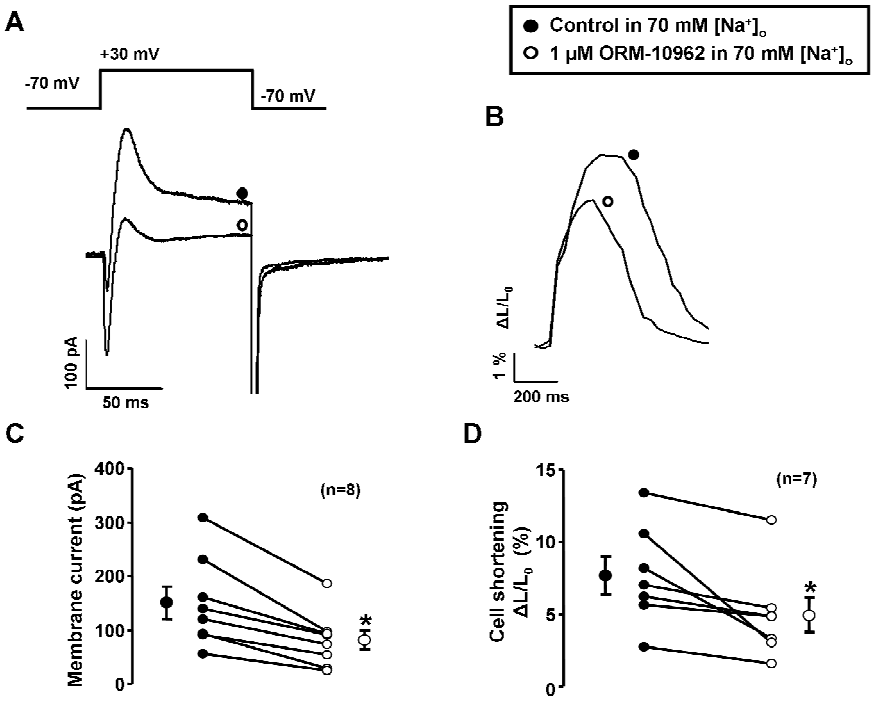

Figure 6.: Effects of 1 µM ORM-10962 on outward NCX current (A, C) and CS (B, D) in the presence of low (70 mM) $[Na^+]_o$. *Cells were stimulated at 1 Hz under voltage clamp conditions using voltage steps to +30 mV for 100 ms arising from the holding potential of -70 mV. Superimposed recordings of membrane currents (A) and CS (B), obtained in the presence and absence of ORM-10962. The corresponding individual and average values are presented in panels C and D. Membrane current was measured at the end of the depolarizing pulse. Open symbols represent results obtained in the presence of ORM-10962, closed symbols are the corresponding controls. Symbols and bars are mean ± SEM values, asterisks denote significant changes induced by exposure to ORM-10962 in 8 and 7 myocytes, respectively.*

3.5. Primary inhibition of forward NCX and secondary decrease of L-type calcium current in response to ORM-10962 results in a net positive inotropic effect

Shifting the NCX balance toward to forward mode activity was the aim of the next set of experiments. I_{Ca} was measured at 0 mV, before this, fast Na^+ current was inactivated at -40 mV by a prepulse. Above mentioned experimental settings together with elevation of $[Ca^{2+}]_i$ by a presence of 200 nM forskolin, were applied to reduce reverse and enhance forward mode NCX activity. **Figure 7A** shows representative traces of I_{Ca} and NCX tail current recorded before and after application of 1 µM ORM-10962 under these conditions. Important to note that after administration of 1 µM ORM-10962 we found considerable reduction in I_{Ca} amplitude shown in **Figure 7C** (from -948±124 to -613±91 pA, $p<0.05$, n=10), while the concomitant suppression of inward NCX tail current is an appropriate indicator of forward

mode NCX inhibition (**Figure 7D**). Important to note that, in contrast to other studies where non selective NCX inhibitors were used, any negative inotropic effect with NCX compound was not observed in these experiments. In fact, the reduced I_{Ca} was accompanied by a strong increase in CS (from 4.7±1.2 to 7.6±1.4 %, $p<0.05$, n=10) as presented in **Figure 7B-E**.

These data may indicate the observed decay in peak I_{Ca} is presumably not a consequence of a direct inhibition, but could be a result of the regulation of increased SR Ca^{2+} release via its Ca^{2+} dependent inactivation [53].

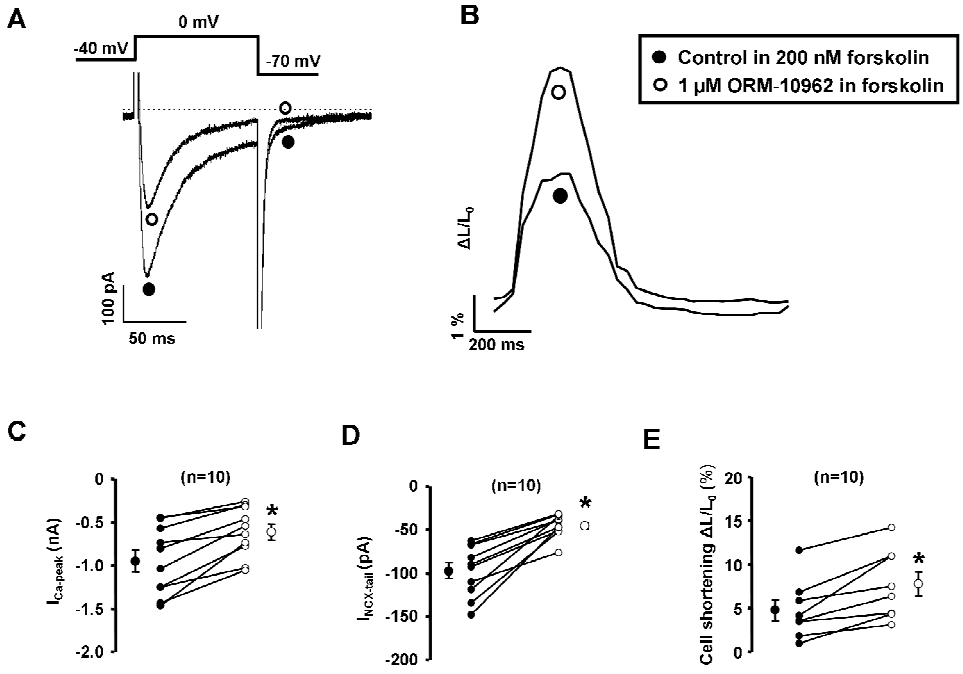

Figure 7.: Effects of 1 µM ORM-10962 on peak L-type Ca^{2+} current (A, C), inward NCX tail current (A, D) and CS (B, E) in the presence of 200 nM forskolin. *In these experiments the cells were paced at 1 Hz using voltage steps to 0 mV (lasting for 100 ms and each preceded by a prepulse to -40 mV) arising from the holding potential of -70 mV. A, B: Superimposed records of the membrane currents (A) and CS (B) obtained in the presence and absence of ORM-10962. Individual and average results are presented in panels C-E. Open symbols represent results obtained in the presence of ORM-10962, closed symbols are the corresponding controls. Symbols and bars are mean ± SEM values, asterisks denote significant changes induced by the superfusion with ORM-10962 in 10 cells.*

3.6. Decrease of the L-type Ca^{2+} current is dependent on the presence of SR Ca^{2+} release

The possible effect of ORM-10962 on the I_{Ca} has crucial importance, since the previous NCX inhibitors exerted some degree of I_{Ca} block in the concentration range corresponding to 70-80% of NCX inhibition [47].

The observed I_{Ca} inhibition could be considered either direct channel suppression or an indirect, Ca^{2+} dependent inhibition by elevated intracellular Ca^{2+}. In order to address this problem the effect of ORM on I_{Ca} was investigated independently from SR release. Therefore a caffeine superfusion was applied to empty the SR and after this voltage pulses were used for measuring I_{Ca}, in the absence and presence of ORM-10962. When SR is empty, the first I_{Ca} kinetic is not influenced by Ca^{2+} release, therefore the possible indirect effects of ORM-10962 is negligible. We found no differences between the amplitudes of the first post-caffeine I_{Ca} traces which were recorded in the presence and absence of ORM-10962 (**Figure 8.A-C**).

Under steady-state conditions - as the SR was gradually refilled - I_{Ca} amplitude gradually decreased trace by trace, however this reduction was significantly greater in the presence of ORM-10962 **(Figure 8.C**). These results indicate that ORM did not influence I_{Ca} kinetics in the absence of SR Ca^{2+} release or, in other words, direct I_{Ca} suppression was not observed in our experiments. In contrast, during SR refilling the indirect Ca^{2+} dependent inhibition was markedly enhanced on peak I_{Ca} current. In order to further strengthen these results we measured I_{Ca} in the presence of 1 μM ORM-10962 using an internal solution containing 10 mM BAPTA a well-known Ca^{2+} chelator. Therefore any shift in the $[Ca^{2+}]_i$ in the vicinity of Ca^{2+} channels was prevented by this pipette solution. **Figure 8.D** shows a representative pair of I_{Ca} traces in the presence of internal BAPTA before and after exposure to 1 μM ORM-10962 which supported no direct inhibitory effect of ORM-10962 on I_{Ca}.

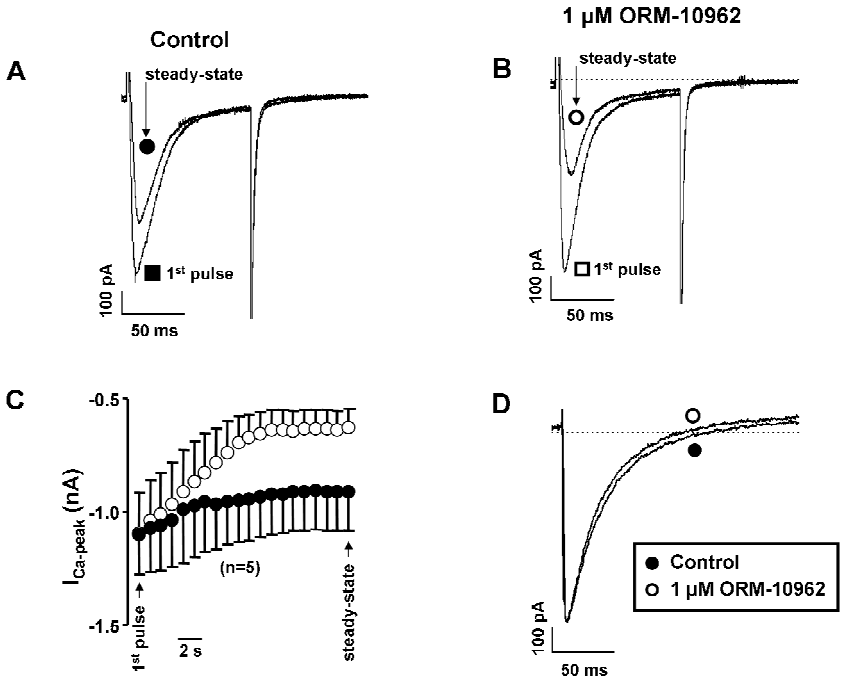

Figure 8.: Effects of 1 μM ORM-10962 on peak I_{Ca}. Repetitive stimulation was applied under voltage clamp conditions after depletion of Ca^{2+} content of the SR using a caffeine pulse. *A, B: Superimposed I_{Ca} records obtained at the beginning (1st pulse, squares) and the end (steady state, circles) of a repetitive stimulation train initiated immediately after the complete depletion of SR. Average results are presented in panel C, where symbols and bars indicate mean ± SEM values obtained in 5 myocytes. D: Superimposed representative I_{Ca} records obtained using 10 mM BAPTA-containing pipette solution before and after exposure to 1 μM ORM-10962. Note the identical time course of the I_{Ca} traces when $[Ca^{2+}]_i$ was effectively buffered by BAPTA. Open and closed symbols represent results obtained in the presence and absence of ORM-10962, respectively.*

3.7. Elevated driving force of the forward NCX partially maintains the Ca^{2+} efflux after NCX inhibition

The positive inotropic effect of the forward mode NCX inhibition may mean that the $[Ca^{2+}]_i$ is elevated, which at the same time provides increased driving force for the forward NCX. In this case the increased driving force may partially balance the reduced NCX capacity after ORM application, which may be manifested as a decrease of the apparent inhibition of inward NCX current, with a possible consequence of limited Ca^{2+} accumulation.

This point was examined further with ORM-10962, taking advantage of the fact that there are two ways to measure the inward NCX current that corresponds to the forward mode activity of NCX. *Firstly*, when NCX removes Ca^{2+} from the cell after maximal SR Ca^{2+} release during the caffeine superfusion an integral of the generated current is an accepted method for

estimation of the SR content, furthermore under this setting the forward NCX activity could be measured by analysis of the peak inward current. In that case the forward NCX in the presence of ORM-10962 is faced with a significantly increased $[Ca^{2+}]_i$, since the elevated SR Ca^{2+} content (see above) is combined with a reduced rate of Ca^{2+} removal. *On the other hand*, inward tail currents recorded upon abrupt back step to the holding potential during normal Ca^{2+} release are also assumed to be mediated by forward mode operation of NCX [4]. In our experiments the measurements of NCX tail currents was applied immediately after emptying of SR by caffeine pulse. This means that measurements in the absence and presence of ORM-10962 the forward NCX faced with similar driving force, since the magnitude of Ca^{2+} influx via I_{Ca} for the first few pulses is practically the same because there is no Ca^{2+} transient which would be able to modulate the Ca^{2+} entry. **Figure 9**, demonstrates the measurement of the peak of I_{NCX} at high $[Ca^{2+}]_i$ after caffeine pulse, and NCX tail currents during voltage pulses on the same cell. Currents were recorded upon repolarization following 0 mV test pulses together with recorded currents after the termination of caffeine superfusion. Significantly increased cell shortening and transient were detected indicating elevated $[Ca^{2+}]_i$ shown at (**Figure 9B and C**). Analysis of relative forward mode NCX blockade revealed that the apparent NCX block induced by 1 μM ORM-10962 was significantly weaker (28.5±4.5% *vs* 50.5±4.4 %, $p<0.05$, n=6), when NCX block was developed in the presence of high $[Ca^{2+}]_i$ after caffeine pulse, compared with low $[Ca^{2+}]_i$ obtained from inward current (**Figure 9A**). During this protocol the corresponding average $[Ca^{2+}]_i$ values were 659±40 and 257±32 nM (n=5), suggesting an inverse linear relationship between the apparent inhibition and the actual $[Ca^{2+}]_i$. Considering that, under high $[Ca^{2+}]_i$ conditions the measured inward NCX current might be contaminated with Ca^{2+}-induced Cl^- current, some of these experiments were repeated in the presence of 200 μM DIDS. In the repeated experiments there were no differences of the apparent NCX inhibition.

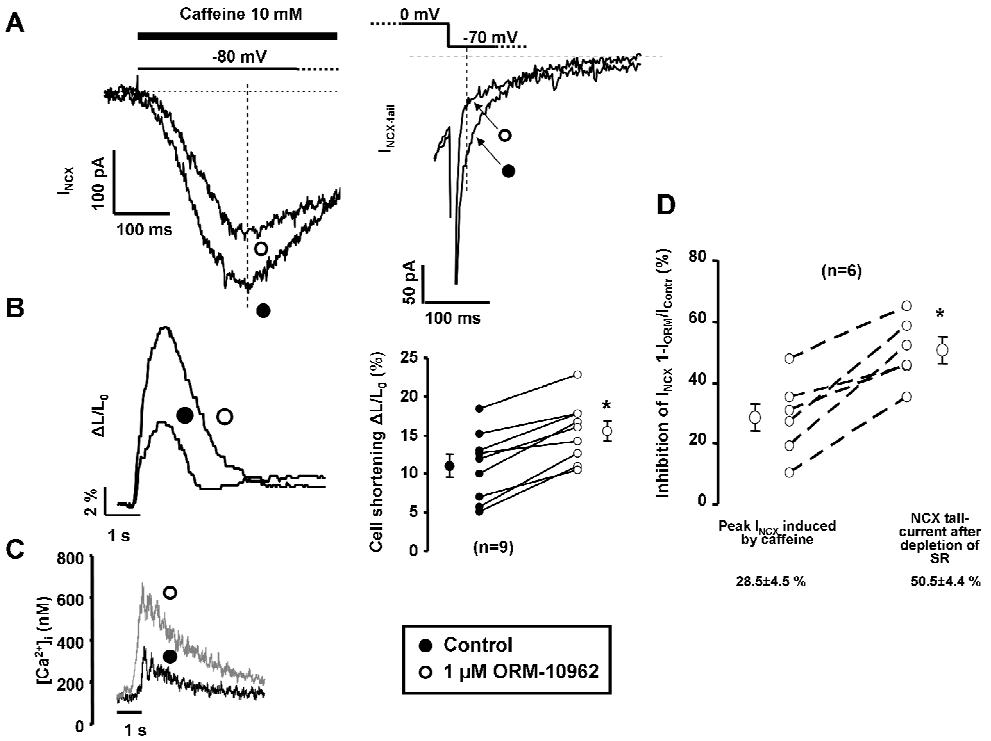

Figure 9. : Apparent inhibition of the inward NCX-current by 1 µM ORM-10962 is attenuated by higher $[Ca^{2+}]_i$ levels. *A: Representative traces of inward NCX-currents evoked by total SR Ca^{2+} release in response to 10 mM caffeine (left) and INCX tail currents recorded without SR Ca^{2+} release in response to voltage pulses applied after caffeine (right) in the absence (filled symbols) and presence (open symbols) of 1 µM ORM-10962. Panel B shows the corresponding representative CS curves (left) and the individual and average effects of ORM-10962 on CS obtained in 9 myocytes (right). Panel C displays a separate experiment where the effect of ORM-10962 is shown on $[Ca^{2+}]_i$ transient recorded using the protocol above. D: Apparent degree of NCX inhibition as estimated by the reduction of peak NCX currents (left) and tail currents (right) compared in 6 myocytes. Dashed lines indicate corresponding data. Open and closed symbols represent results obtained in the presence and absence of ORM-10962, respectively. Symbols and bars are mean ± SEM values, asterisks denote significant differences between control and OMR-10962-treated values.*

3.8. Selective NCX inhibition reverted the hypokalaemia induced positive inotropy

In order to investigate the effect of NCX inhibition on hypokalaemia induced alteration of cell shortening, a 2.7 mM KCl-contained Tyrode's solution was applied after normal condition at 1 Hz pacing frequency. Under this setting we found significant increase of cell shortening (2.3±1% → 12.3±14%, n=22, $p<0.05$; **Figure 10A**). The subsequently used ORM-10962 reverted the hypokalaemia induced increase in cell shortenings (12.3±14% → 6.12±11%, n=22 respectively, $p<0.05$; **Figure 10B-C**). The extra-beats, contraction waves were also

counted during the experiments which were completely absent under normal condition. Under hypokalaemic settings significant number of extra contractions appeared (13.8±2 extras/ 8 minutes; n=22, p<0.05) (**Figure 10A**). Similarly to the results of cell shortening the selective NCX inhibition decreased the number of extra beats (7.5±1.8 extra/8 min; n=22, p<0.05; **Figure 11A**).

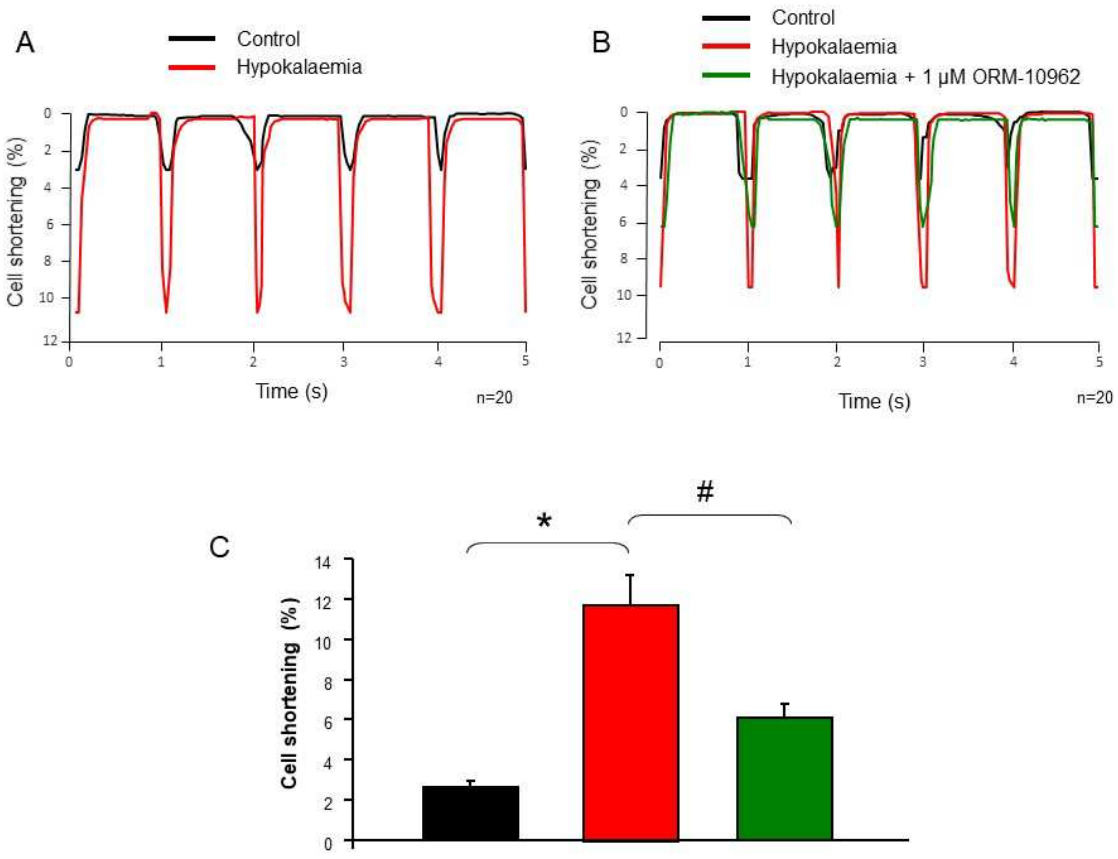

Figure 10. : The hypokalaemia induced increased cell shortening in hypokalaemic condition is reduced by 1 µM ORM-10962. *The hypokalaemic solution significantly increased the cell shortening (A). The subsequently applied inhibition of NCX by 1 µM ORM-10962 reduced the magnitude of cell shortening (B). Bar graph (C) shows the percentage (%) of the changes of cell shortening in normal (black column), hypokalaemic condition (red column) and in the presence of 1 µM ORM-10962 (green column).*

In the presence of hypokalaemic solution the cells often exerted paroxysmal "fibrillation" episodes meaning an irregular, high frequency fluttering instead of regular contraction which were termed as 'contraction waves' in this study. Since these waves showed transient nature, we calculated the cumulative duration of the episodes during and experiment and compared

these between control and ORM-10962 treated conditions. Under normal settings no contraction waves were detected however in hypokalaemic condition they were present for a period (15.3±4 sec/8 min, n=22, $p<0.05$, **Figure 11B**). In the presence of ORM-10962 the duration of contraction waves were significantly reduced (8.36±2 sec/8 min; n=22, $p<0.05$; **Figure 11B**).

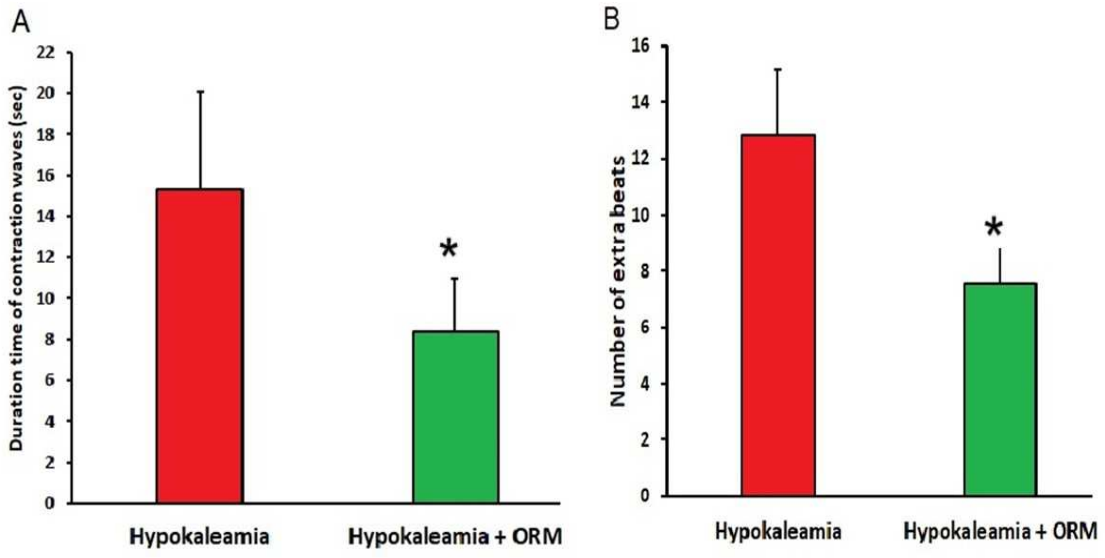

Figure 11. : Extra beats and contraction waves in hypokalaemic condition are reduced by 1 µM ORM-10962. *A: In hypokalaemic condition (red column) a significant number of extra beats appeared which were suppressed in the presence of 1 µM ORM-10962 (green column). B: The average total duration of contraction waves were substantial in hypokalaemic condition (red column). The application of selective NCX blockade (green column) by 1 µM ORM-10962 markedly decreased the duration of the high frequency episodes.*

4. DISCUSSION

In the present study various effects of novel NCX inhibitors (GYKB-6635 and ORM-10962) were assessed. Emphasis was laid on the following:

(1) Novel NCX inhibitor GYKB-6635 effectively inhibits both mode of the NCX current.

(2) ORM-10962 has no direct blocking effect on L-type Ca^{2+} channels in the lack of SR Ca^{2+} release but strongly suppresses I_{Ca} under conditions of normal Ca^{2+} cycling.

(3) The experimental conditions strictly determined the inotropic action of NCX: the inhibition of previously activated reverse mode resulted in negative inotropy, while NCX blockade during forward mode facilitation caused mild positive inotropy.

(4) The inhibitory action of forward NCX suppression by ORM-10962 is functionally asymmetrical: the shift in Ca^{2+} handling appears to be more marked in the reverse than in the forward mode NCX blockade. Possibly Ca^{2+} accumulation caused by forward mode NCX inhibition is effectively attenuated by the autoregulation of Ca^{2+} handling and also by the sustained inducibility of NCX by the elevated $[Ca^{2+}]_i$, which add an increased driving force for the forward operation.

(5) ORM-10962, presumably by reduction of the reverse mode NCX function, reverted the $[Ca^{2+}]_i$ overload induced by experimental hypokalaemia.

4.1. 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}) [54]. 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 [23, 47]. 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.

4.2. 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 $[Ca^{2+}]_i$ [55].

4.3. 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 [24], 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 (**Fig.4**). 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.

4.4. 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 [24]. 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 (**Fig. 5A and C**). 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 [56]. 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 [57].

4.5. Possibility of an asymmetrical NCX blockade

Even though some evidences demonstrating that mode selective NCX inhibition may occur under certain experimental conditions [58], theoretical considerations do not support the possibility of transport mode dependent inhibition [59].

Previous papers demonstrated that KB-R7943 and SEA0400 preferentially block the reverse mode NCX [28, 47]. In our previous studies, ORM-10103 or ORM-10962 exerted balanced inhibitory effect between reverse and forward mode [23, 24], 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 [60]. When SEA0400 was applied to block NCX in a previous study [46], some evidence was found which supporting our theory.

4.6. 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.

4.6.1. Adaptive changes in Ca^{2+} influx following NCX inhibition

The autoregulation of the Ca^{2+} handling is a simple feed-back mechanism to control the $Ca^{2+}{}_i$ of the cell by regulating the Ca^{2+} efflux and influx [9]. The basis of the autoregulation is the Ca^{2+}-dependent inhibition (CDI) of I_{Ca} which is highly limited when SR Ca^{2+} release is decreased providing larger Ca^{2+} influx to increase the SR Ca^{2+} content. In the case of high SR Ca^{2+} and large release, the CDI restricts the Ca^{2+} influx to protect the cell from Ca^{2+} overload, at the same time the Ca^{2+} efflux is enhanced. It is important that during steady-state, the autoregulation maintains the influx and efflux in balance [9]. Since SERCA and NCX are competing for Ca^{2+} during relaxation, significant increase in Ca^{2+} sequestration into the SR can be expected after forward mode NCX blockade as causing stronger Ca^{2+} release. Since inactivation of L-type Ca^{2+} channels is under tight control of local Ca^{2+} [8, 61] the increased SR Ca^{2+} release may limit the Ca^{2+} influx through Ca^{2+} channels, facilitating the development of a new steady state reached at lower rates of Ca^{2+} efflux and influx. Indeed, in our experiments the increased SR Ca^{2+} content after ORM-10962 administration was paralleled with a considerable decrease in Ca^{2+} influx, as the decreased amplitude of I_{Ca} reported. It is important to mention here, that this reduction of I_{Ca} magnitude was observable only when Ca^{2+} handling was not buffered indicating indirect suppression of the current. The concept of autoregulation may help to interpret the slight positive inotropy under baseline conditions. Although in our Ca^{2+} transient measurements we could measure neither Ca^{2+} influx nor the Ca^{2+} content of the SR, we can speculate that the capacity of the autoregulatory mechanisms may be high enough to largely compensate for the reduced Ca^{2+} efflux [62] causing only a marginal shift in the Ca^{2+} balance. However, when Ca^{2+} cycling and the forward NCX is augmented by forskolin (**Fig. 7**), the elevated local Ca^{2+} might compromise the original capacity of autoregulation, thus the reduced Ca^{2+} efflux after NCX inhibition cannot be

balanced, which results in a net Ca^{2+} accumulation with positive inotropic consequences. Nevertheless, this issue requires further investigation.

4.6.2. Possible contribution of alternative Ca^{2+} removal pathways

The involvement of alternative Ca^{2+} removal pathways such as sarcolemmal Ca^{2+} ATPase could be also explained in the Ca^{2+} removal mechanism during NCX inhibition. However it was described earlier that the capacity of sarcolemmal ATPase is limited and may be involved in the fine tuning of diastolic Ca^{2+} level instead of major role in the extrusion of systolic Ca^{2+} [63]. However since its enhancement by the elevated submembrane $[Ca^{2+}]_i$ which may compensate for the decay in forward mode NCX activity, cannot be ruled out and may explain the lack of Ca^{2+} overload and cell death following NCX blockade in healthy canine cardiac cells.

4.6.3. Preserved inducibility of forward mode NCX by elevated $[Ca^{2+}]_i$

The extent of forward mode NCX suppression can be estimated from inward NCX current traces monitored in the absence and presence of ORM-10962. The rapid application of caffeine deliberates the total amount of SR Ca^{2+}, which is subsequently extruded from the cytoplasm by forward mode NCX activity. The integral of the developed inward current is used to estimate the SR Ca^{2+} content and the magnitude of forward NCX. After analysis of our experiments it was clear that ORM-10962 is unable to block forward mode NCX completely (**Fig. 9A**), and the degree of inhibition calculated from peak, caffeine-induced NCX current traces reached only approx. 30 % - much less than the 80 % blockade expected from the experiments in Ca^{2+} buffered cells using similar drug concentrations [23, 24]. Since the degree of caffeine-induced contracture, and consequently the peak $[Ca^{2+}]_i$ values, were significantly elevated in the presence of ORM-10962, it is obvious that the uninhibited fraction of NCX functions in the presence of a highly elevated driving force. The degree of the apparent inhibition may therefore be restricted by the increased driving force for Ca^{2+} removal, implicating that the inhibition of NCX by ORM-10962 is a dynamic and $Ca^{2+}{}_i$ dependent phenomenon. The hypothesis of dynamic Ca^{2+} dependent NCX blockade is supported by inhibition of NCX tail currents recorded immediately after depletion of SR Ca^{2+} content by caffeine. Since there is no Ca^{2+} release during the initial post-caffeine stimulation

pulses, the elevation of $[Ca^{2+}]_i$ may be solely due to Ca^{2+} influx mediated by I_{Ca}. It is practically exerts the same magnitude during the first few I_{Ca} traces which is not influenced by the ORM-10962 since in the absence of Ca^{2+} release from the SR there is no substantial increase in the driving force for the forward NCX. In this case the ORM-10962 exerted ~ 50 % reduction of the NCX tail current, yielding a more pronounced NCX inhibition compared to the 30 % inhibition obtained at high $[Ca^{2+}]_i$ levels evoked during caffeine stimulus. Thus, the partial escape of NCX from the inhibition in the presence of high $Ca^{2+}{}_i$ levels may cause an asymmetrical inhibition of the NCX, with decreased consequences of forward mode inhibition. This may implicate that the NCX inhibition by ORM-10962 may be manifested as a reduced sensitivity of the forward NCX to the activator Ca^{2+}, meaning that the "activation curve" of forward NCX is shifted toward higher Ca^{2+} levels in the presence of ORM-10962. Therefore, inward NCX current can be activated further in the presence of ORM-10962, however it requires a considerably higher level of $[Ca^{2+}]_i$. This may also implicate, that the above mentioned higher $Ca^{2+}{}_i$ level could be developed after NCX inhibition decreasing the apparent degree of inhibition. These results were consequent to our previous findings with SEA-0400 [46].

4.7. 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 (**Fig. 10**). 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 (**Fig. 11**). 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 [43, 49] we found marked increase in Ca^{2+} transient and cell shortening/contraction after NCX inhibition, and we failed to reproduce this effect on dog [46] and rabbit [43] 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 (10% *vs* 30-40% [64, 65]) 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 [56]. 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 [49] and marginal effect was observed in the case of guinea-pig [45], rabbit [43], and dogs [46]. 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.

4.8. Therapeutic implications: NCX inhibition as a possible positive inotropic intervention

This study suggests that in intact myocytes the functional consequence of NCX inhibition by ORM-10962 is an asymmetrical blockade showing a dominant effect on reverse mode NCX activity. When Ca^{2+} influx via reverse mode NCX significantly contributes to Ca^{2+} balance the NCX inhibition will be substantial and a net negative inotropic effect is a result of decreased cellular Ca^{2+} content.

The positive inotropy due to forward mode NCX inhibition may become apparent during conditions when reverse mode NCX is suppressed and Ca^{2+} cycling is stimulated independently from the Ca^{2+} influx through reverse mode NCX. In heart failure the NCX reverse activity is increased [66, 67] and the NCX inhibition by ORM-10962 may block primarily the reverse mode NCX. But reverse mode is a rescue mechanism to maintain the cellular Ca^{2+} load [66, 67]. This is not necessarily balanced by the decreased Ca^{2+} loss, since this latter appears to be a less pronounced consequence of NCX inhibition. Thus the possibility of improving cardiac inotropy by pharmacological NCX inhibition requires further detailed studies in myocytes obtained from failing hearts.

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6. ACKNOWLEDGEMENT

I am especially thankful to my PhD supervisors **Dr. Károly Acsai** and **Dr. Norbert Nagy**, for personal guidance, for their professional advice, continuous support of my work and for introducing me to the research field of cardiac cellular electrophysiology.

I am very appreciating to **Professor Julius Gy. Papp MD, DSc, academian**, for his support and his comments which advised me constructive criticism and suggestions for the further research. I am also grateful to **Professor András Varró MD, DSc** for providing me the opportunity for conducting research as PhD student at the Department of Pharmacology and Pharmacotherapy, University of Szeged.

This work was supported by the grants from the National Research Development and Innovation Office (OTKA PD-125402, NN-109994, NK-104331, K-119992, GINOP-2.3.2-15-2016-00006 and GINOP-2.3.2-15-2016-00012), the National Office for Research and Technology- Baross and Ányos Jedlik Programmes (REG-DA-09-2-2009-0115-NCXINHIB and NKFP_07_01-RYT07_AF), the Ministry of National Education - New National Excellence Program of the Ministry of Human Capacities (UNKP-16-3 - IKT/147-1787/8/2016-ÖSZT-95), HU-RO Cross-Border Cooperation Programmes (HURO/1001/086/2.2.1_HURO-TWIN), LIVE LONGER EFOP-3.6.2-16-2017-00006. and the Hungarian Academy of Sciences. I especially acknowledge the professional support offered by Orion Pharma Finland by supplying ORM-10103 and ORM-10962 compounds.

7. ANNEX

Publications related with the thesis