Prevention of intracellular calcium overload by blocking NCX

PhD Thesis

Judit Szepesi, Msc

Department of Pharmacology & Pharmacotherapy

Faculty of Medicine,

University of Szeged

Szeged, Hungary

Szeged

2015

PUBLICATION LISTS

a.)Publication list related to PhD topic

I. *Szepesi J, Acsai K, Sebok Z, Prorok J, Pollesello P, Levijoki J, Papp JG, Varro A, Toth A*
Comparison of the efficiency of Na+/Ca2+ exchanger or Na+/H+ exchanger inhibition and their combination in reducing coronary reperfusion-induced arrhythmias
JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 66:(2) pp. 215226. (2015)
IF: 2.720

II. *Nagy N, Kormos A, Kohajda Z, Szebeni A, Szepesi J, Pollesello P, Levijoki J, Acsai K, Virag L, Nanasi PP, Papp JGy, Varro A, Toth A*
Selective Na+/Ca2+ exchanger inhibition prevents Ca2+ overload induced triggered arrhythmias.
BRITISH JOURNAL OF PHARMACOLOGY 171:pp.5665-5681(2014)
IF.: 4,99

b.) Conference presentations related to PhD topic

Szepesi J, Sebők Zs, Tóth A
Effect of NCX and NHE inhibition on the development and duration of postischemic arrhythmias (NCX és NHEgátlás hatása posztiszkémiás aritmiák kialakulására és időtartamára)
CARDIOLOGIA HUNGARICA 42:(Suppl.A) p. A37. (2012)

Szepesi J
The effect of combined NCX1 and NHE1 blockade in acute ischemia/reperfusion injury in rat
SEMINAR FOR YOUNG RESEARCHERS The physiology, pathophysiology and pharmacology of the cardiovascular system (2012)

Posters

Szepesi J

The effect of NCX and NHE inhibition on the development and duration of post-ischemic arrhythmias

- *Sudden Cardiac Death & Cardioprotection conference and advanced workshop (2012)*
- *EHRA EUROPACE 2013 –The meeting of the european heart rhythm association (2013)*

INTRODUCTION

Cardiovascular diseases are the leading causes of death all over the world. Heart failure (HF) and arrhythmias are the most serious conditions likely resulting in death. The causes of HF include of ischaemic coronary disease, myocardial infarction, chronic hypertension and valvular diseases. Coronary reperfusion therapy is a widely used intervention for the management of acute myocardial infarction (AMI). However, restoration of blood flow to previously ischaemic myocardium results in the so-called ischaemia/reperfusion (IR)-injury, which can be manifested as myocardial necrosis, arrhythmia, myocardial stunning and endothelial- and microvascular dysfunction including the no-reflow phenomenon. The mechanisms of the ischaemia/reperfusion injury consists of several pathways. Recently, there's increasing evidence for an important role in IR injury on hypercontracture induced by calcium overload or low ATP level. Reperfusion of the heart after ischaemia may lead to lethal arrythmias. In humans, the most common reperfusion arrhythmia is an accelerated idioventricular rhythm. However, ventricular tachycardia and ventricular fibrillation remain the most important causes of sudden death following spontaneous restoration of the coronary flow. The development of cardiac arrhythmias require concomitant existence of a *trigger* (i.e.: extrasystoles, generated by large enough membrane potential oscillations, usually induced by perturbations in $[Ca^{2+}]_i$ handling, leading to $[Ca^{2+}]_i$ overload) and a *substrate* (i.e: large enough APD dispersion between adjacent cells, typically caused by an uneven reduction in the efficacy of AP repolarization). In normal conditions $[Ca^{2+}]_i$ is under a tight control due to a delicate balance between Ca^{2+} fluxes. Therefore, $[Ca^{2+}]_i$ overload is prevented and triggered events cannot occur. Furthermore, the fast propagation of the action potencial and the homogenous repolarization (i.e.: small APD dispersion between adjacent cells) precedes circular reentry.

Crucial role of the severe disturbances in the intracellular ionic homeostasis (including changes in $[Na^+]_i$, $[K^+]_i$, $[H^+]_i$, $[Ca^{2+}]_i$) in the initiation and progression of potentially lethal cardiac arrhythmias has been demonstrated in a plethora of studies. Prognoses of these arrhythmias are poor with a high rate of mortality, due to their complex and not fully understood pathomechanisms and the consequent sub-optimal medical treatment. Since increased NCX activity is considered as a major cause of both the ischaemia induced tissue injury and postischaemic reperfusion arrhythmias, a beneficial effect of NCX inhibition against these pathological states seems highly feasible and is suggested by experts in the field.

Intracellular Ca^{2+} homeostasis and excitation-contraction coupling

Ca^{2+} is a very important intracellular messenger. Calcium signaling is essential for many physiological processes, including muscle contraction, cell mobility, fertilization, exocytosis, and apoptosis. One of the major players in regulating intracellular Ca^{2+} is the Na^{+}/Ca^{2+} exchanger (NCX). In cardiac excitation - contraction (E–C) coupling, a small amount of Ca^{2+} first enters through the L-type Ca^{2+} channel (LTCC) during membrane depolarization. This Ca^{2+} influx triggers a larger Ca^{2+} release through the Ca^{2+} release channels of the sarcoplasmic reticulum (SR), referred to as ryanodine receptors (RyRs). The gain of excitation-contraction (EC) expresses the relationship between SR Ca release flux and the Ca current that triggers it and is an expression of its efficiency.

Ca removal from the cytoplasm initiates the myocyte relaxation. There are two major routes of removal: SR Ca ATPase (SERCA2a) pumps Ca back into the SR and is regulated by phospholamban (PLN); the other is a subsequently transsarcolemmal Ca^{2+} removal through NCX, which operates in its forward mode when Ca^{2+} is high. NCX is a bi-directional transporter working in either forward and reverse mode depending on the electrochemical gradients of the substrate ions. Under physiologic conditions, forward NCX removes majority of Ca that entered the cell through LCCs and reverse NCX, in order to maintain cellular Ca balance. The forward mode that removes calcium is coupled with sodium influx in $3Na^{+}$: $1Ca^{2+}$ stoichiometric ratio. Under physiological conditions and especially in pathological conditions such as ischemia/ reperfusion the NCX allows Ca^{2+} entry and sodium extrusion, called reverse mode operation.

Cardiac NCX structure and function

The cardiac NCX1 consists of 970 amino acids with a molecular mass of 110 kDa. Presently, in the latest version, the Na^{+}/Ca^{2+} exchanger contains 9 transmembrane segments, 5 from the NH2 terminus up to the large intracellular loop and 4 from that loop up to the COOH terminus.

In cardiac muscle, one of the isoforms of the sodium calcium exchanger, NCX1, represents a major system of extrusion of Ca^{2+} that enters the cell by L-type Ca^{2+} channels (Cav1.2). Like other Ca^{2+} transport systems which are involved in excitation-contraction coupling (Cav1.2 and Ca^{2+} release channels of SR), NCX1 is regulated by intracellular Ca^{2+}.

In cardiac myocytes, function of the Na^{+}/Ca^{2+} exchanger belongs to the most important mechanisms of the Ca^{2+} homeostasis, and displays a major contribution to the regulation of the Ca^{2+} level during the cardiac excitation-contraction coupling. The major function of the

NCX in the heart is the extrusion of Ca^{2+} from cytoplasm during relaxation and diastole. The major source of Ca^{2+} triggering the Ca^{2+} cycle is the L-type Ca^{2+} current which flows into the cell at the beginning of the action potential. In this phase of the action potential, the NCX can contribute to the Ca^{2+} influx into the cell since the membrane potential is positive and the intracellular Ca^{2+} level is low. However, when the intracellular Ca^{2+} level increases at the beginning of the Ca^{2+} transient due to the Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum, the NCX turns into its forward mode operation, thereby contributing to the extrusion of Ca^{2+} from the cell. Relaxation of the Ca^{2+} transient is a result of the activities of tree competing mechanisms.

Intracellular pH Regulation in the Cardiac Cell

Cardiac myocytes expresses the apparatus for pH_i regulation by which an acute displacement of pH_i is compensated within minutes. The recovery of pH_i is mediated by sarcolemmal ion transport proteins that move H^+, OH^- or HCO_3^- ions across the membrane. It should be noted that influx of one HCO_3^- anion, by neutralising an intracellular H^+ ion , raises pH_i by the same amount as the direct efflux of one H^+ ion. These ion transport steps are thus referred to as H^+-equivalent ion movements. At least five generic types of H^+-equivalent transport protein have been identified functionally in the mammalian ventricular myocyte. Na^+/H^+ exchange (NHE) and Na^+/HCO_3^- cotransport (NBC) mediate acid-extrusion while Cl^-/HCO_3^- exchange (CBE) and Cl^-/OH^- exchange (CHE) mediate acid loading. A lactate–H^+ cotransport (monocarboxylate transporter, MCT) operates in either efflux or influx mode, depending on the actual circumstances. At normal physiologic pH, all four regulators have low activity. When intracellular pH rises, the acid loaders are activated, restoring intracellular pH through an influx of acid. When intracellular pH falls, the acid extruders are activated, restoring intracellular pH by removing acid.

Effect of Ischemia on Cellular Sodium and Calcium Homeostasis

Myocardial ischemia occurs when the energy demands of the cardiac tissue are not met by the available energy substrates. Deprivation of oxygen and nutrient supply results in a series of biochemical and metabolic changes within the myocardium. The absence of oxygen halts oxidative phosphorylation, leading to mitochondrial membrane depolarization, ATP depletion, and inhibition of myocardial contractile function. In the absence of oxygen, cellular metabolism switches to anaerobic glycolysis, resulting in the accumulation of lactate, which reduces intracellular pH (to <7.0). The acidic conditions during ischaemia prevent the

opening of the MPTP and cardiomyocyte hypercontracture at this time. The intracellular accumulation of protons activates the Na^+-H^+ ion exchanger, which extrudes protons from the cell in exchange for Na^+ entry. The lack of ATP during ischemia ceases function of the Na^+-K^+ ATPase, thereby exacerbating the intracellular Na^+ overload. In response, the reverse activation of the NCX results in intracellular Ca^{2+} overloading as the cell tries to extrude Na^+. This contributes to intracellular Ca^{2+} overload and damages the cell membrane. Reperfusion and reactivation of the NHE result in washout of lactic acid, resulting in the rapid restoration of physiological pH, which releases the inhibitory effect on MPTP opening and cardiomyocyte contracture. The restoration of the mitochondrial membrane potential drives calcium into the mitochondria, which can also induce MPTP opening.

Pharmacology of NCX

Potent and selective blockers of NCX activity would be extremely useful for clarifying the precise functions of NCX. Until recently, however, this possibility was hampered in case of NCX because of the lack of potent and highly specific inhibitors.

Because NCX moves ions and charge in either direction, mode-specific inhibitors of NCX, if available, may have high therapeutic potential. For example, specific blocking of excessive Ca^{2+} influx via reverse-mode NCX activity may reduce Ca^{2+} overload due to cardiac glycoside toxicity or to ischemia and reperfusion in the heart and other tissues. Nowadays, the two most selective and widely used NCX inhibitors in the literature are the aniline derivative SEA0400 and KB-R7943. Intriguingly, KB-R7943 exerts a preferential effect on reverse-mode (Ca^{2+}-influx mode) NCX activity. However, the selective blocking of Ca^{2+} influx via NCX is observed irrespective of the presence or absence of extracellular Ca^{2+}. In cardiac sarcolemmal vesicles, the XIP peptide and a Ca^{2+} channel blocker, nicaldipine, depressed the rate of Na^+-dependent Ca^{2+} uptake much more potently than that of Na^+-dependent Ca^{2+} efflux. Although the data suggest a significant difference in the properties of NCX1 in the forward and reverse modes, the underlying molecular mechanism for this peculiar directional specificity is not clear.

The selective inhibitor of Na^+/Ca^{2+} exchange SEA0400 was up to 100 times more potent than KB-R7943. Some synthetic peptides are also effective NCX inhibitors. The XIP peptide derived from the primary sequence of cardiac NCX1 decreases the V_{max} of NCX activity. XIP has little effect on Na^+/K^+-ATPase, SR Ca^{2+}-ATPase, or L-type Ca^{2+} currents, and it does not increase membrane conductance when applied to the intracellular surface by use of the excised-patch technique.

Iwamoto and colleagues describe the characterization of a new NCX inhibitor, SN-6, a derivative of KB-R7943. As reported recently for SEA0400, SN-6 seems to act by accelerating Na^+-dependent inactivation, thereby preferentially inhibiting the "reverse" mode transport that occurs with elevated intracellular Na^+ during hypoxia.

In conclusion, although there are several pharmacological agents inhibiting the NCX, the interpretation of results obtained using these compounds is complicated by the concomittant effects on other transport systems or ionic channels.

Cardioprotective Effects of NCX and NHE inhibitor

A reasonable defensive strategy to limit the ischaemia/reperfusion-induced $[Na^+]_i$ accumulation and subsequent $[Ca^{2+}]_i$ overload seems to be a pharmacological inhibition of one or both exchangers. In preclinical studies, NHE inhibition has been shown to be highly effective, especially when applied *before* ischaemia. Based on the promising results of these studies, NHE inhibition has been suggested as a straightforward therapeutic strategy against ischaemia-reperfusion-induced cardiac injuries.

In a range of preclinical studies, especially under moderate ischaemia, reverse mode inhibition of NCX has been found to suppress both $[Na^+]_i$ and $[Ca^{2+}]_i$ overload, hypercontracture, enzyme release and cell damage. Consequently, the inhibition of NCX may have substantial therapeutic potential in the prevention of cardiac ischaemia/reperfusion-induced arrhythmias and injuries. Evaluation of the experimental data on NCX inhibition was, however, seriously hampered by the lack of selective and highly effective NCX inhibitors. This may explain why only NHE inhibitors are used to some extent in clinical practice. NCX inhibitors have never been tested in clinical trials.

In summary, there is convincing evidence suggesting that in *clinical practice,* NHE inhibition, *per se,* fails to prevent or substantially reduce ischaemia/reperfusion-induced arrhythmogenesis. On the other hand, reverse mode NCX inhibition displayed promising cardioprotective effects in animal studies. The present work was based on the assumption, that since the enhanced NHE activity is only *one* factor resulting in cardiac arrhythmogenesis due to the subsequent $[Ca^{2+}]_i$ overload, the inhibition of NHE itself, does not seem to be the *optimal treatment* to reduce the incidence and severity of cardiac arrhythmias. One may also speculate that since the NHE and NCX seem to play a joint key role in the induction of ischaemia/reperfusion-induced $[Ca^{2+}]_i$ overload, the deteriorative effects might be better attenuated by simultaneously inhibiting both the NHE and NCX.

AIMS OF THE STUDY

The primary aim of our studies was the detailed analysis of the cardioprotective effects of NCX and/or NHE blockade against $[Na^+]_i$ rise induced $[Ca^{2+}]_i$ overload and subsequent changes in action potential morphology in experimental models of LQT3 syndrome and ischaemia/reperfusion injury. The major questions were:

- Can SEA0400 or ORM-10103 prevent ATX-II induced changes in AP morphology and APD prolongation?
- Is individual or combined inhibition of NHE and/or NCX protective following regional ischaemia, against arrhythmias, at least partially generated by reperfusion-induced $[Ca^{2+}]_i$ -overload in Langendorff - perfused rat hearts?
- Does NCX and/or NHE inhibition have a protective effect on Ca^{2+} overload induced contracture during and following global ischaemia?

For this purpose, a well-known, well-characterised NHE inhibitor, cariporide, and two NCX inhibitors, the fairly selective SEA0400 and the novel, more selective ORM-10103, were used.

RESULTS

SEA0400 and ORM-10103 fail to eliminate the APD lengthening effect of ATX-II

When SEA0400 and ORM-10103 were applied after ATX-II, eg. after ATX-II significantly lenghtened APD, they exerted no any modulation on the ATX-II –induced APD prolongation. Similarly, when the NCX inhibitors were added as pretreatment, they were unable to prevent the ATX-II-induced APD prolongation. Thus, application of 1 μM SEA0400 either prior to or following the ATX-II treatment had no effect on APD. Similarly to SEA0400, the more selective ORM-10103 was also ineffective against the ATX-II induced APD lengthening. Independently of the sequence of application it failed to prevent or even reduce significantly the APD lengthening effect of ATX-II.

Comparison of the ischaemia-induced contracture's parameters

Contracture, i.e. a sustained shortening and stiffening of the myocardium, can have several causes. In ischaemic myocardium, contracture develops by a rigor-type mechanism.

We compare the time to the peak contracture occuring as a consequence of the ischaemia. Time to peak contracture was significantly increased by the applied drugs compared to the control group, irrespective of whether they were applied alone or in combination.
The amplitude of the contracture was significantly decreased by the drugs during the 25 min ischaemic period. Again, they were equally effective alone and in combination.
We also analysed the contracture developed as a consequence of the reperfusion induced myocardial damage. Again, SEA0400 and cariporide decreased the extent of the contracture significantly compared to the vehicle treated group. Interestingly, contrary to the expectations, combination of SEA0400 and cariporide did not show any increased protection neither in ischaemia nor in the reperfusion-induced contracture.

Measurements of functional recovery

The end-diastolic pressure is the main indicator of the ability of the heart to relax at the end of cardiac cycle. Thus, it is an important parameter for mapping how the myocardial cells damaged and developed the contracture under ischaemic conditions and also during the first minutes of the reperfusion. Functional impairment associated with ischaemia and reperfusion was also assessed by measurements of left ventricular developed pressure (LVDP) and changes in end -diastolic pressure (LVEDP). In the control group effective mechanical activity (LVDP > 8 Hgmm) recovered only in 25% of the hearts – during the first 10 minutes of reperfusion. This value was significantly improved in the treatment groups**.** The rate of effective recovery was 55,5% in the hearts treated with SEA0400, while cariporide increased it to 87,5 %. The recovery rate was 68,7% in the heart with combined SEA0400 and cariporide treatment. The reperfusion caused further damage in the ventricular functions, indicated by the elevated end diastolic pressure and the decreased developed pressure. Among the inhibitors only cariporide was able to demonstrate a significant protection regarding LVDP. Interestingly, the combinated treatment (SEA0400+ CAR) did not provide a greater protection**.** In both treated groups the end-diastolic pressure was significantly lower than in the control group. This cardioprotective effect was maintained during the whole reperfusion

Regional ischaemia

Arrhythmia diagrams

Following a relatively short (10 min) period of regional ischaemia, the most common type of reperfusion-induced arrhythmias evoked in control (CON) rat hearts (treated solely with the vehicle, DMSO) was VF. The antiarrhythmic efficacy of the selective NHE inhibitor,

cariporide, at least in this simple model, was excellent. Compared to the very high incidence of VFs present in the control group, cariporide treatment drastically reduced the incidence and even more the duration of reperfusion-induced arrhythmias. The antiarrhythmic efficacy of the two NCX inhibitors, SEA0400 and ORM-10103, either alone or in combination with cariporide, can hardly be quantitatively estimated by simply looking at the arrhythmia diagram. Nonetheless, it is obvious that none of these treatments has been as effective as cariporide treatment alone. In order to better reveal the relatively moderate but possibly important differences between various treatments, especially for the two types of less severe reperfusion induced arrhythmias (ES and VT), a more detailed, quantitative analysis has been carried out.

Quantitative analysis of the incidence and duration during reperfusion of the sinus rhythm (SR) and the three major types of arrhythmia (ES, VT and VF)

Cariporide, when used alone, significantly increased the overall duration of the SR periods during reperfusion (25.8±2.4 min). All other pharmacological treatments were apparently beneficial, but neither was found to be significant. While in three of the four groups with an NCX inhibitor, the effect was similar (10.26±3.89; 10.57±3.61 and 11.25±4.16 min for SEA, SEA+CAR and ORM+CAR groups, respectively), further to cariporide, the second largest improvement was achieved in the group treated with ORM alone (14.86±4.24 min).

In control hearts, both the average duration (VF_D) (19.39±3.01 min) and incidence (VF_I) (1.35±0.24 min^{-1}) of VF was high.

Compared to the control hearts, cariporide, if used alone, significantly decreased the incidence (0.55±0.24 min^{-1}) and particularly the duration (0.25±0.21 min) of the fibrillatory periods. At the end of reperfusion in this group, none of the 9 hearts was fibrillating. Unlike cariporide, however, the NCX inhibitors, although exerting a moderate beneficial effect, were apparently unable to significantly reduce the incidence or duration of the VF periods. Neither SEA0400 (I: 1.08±0.37 min^{-1}; D: 17.86±4.07 min) nor ORM-10103 (I: 0.80±0.13 min^{-1}; D: 11.57±4.29 min) could markedly decrease the incidence or average duration of the VF periods. From this aspect, the overall anti-VF efficacy of ORM-10103 was slightly more promising. While no apparent difference could be observed in the number of hearts with sustained fibrillation (6/12 (ORM-10103) compared to 7/13 (SEA0400), the duration of the VF periods was moderately lower in the ORM-10103-treated group compared to the SEA0400-treated group.

In control hearts, both the incidence (ES_I) (0.11±0.02 min^{-1}) and duration (ES_D) (0.14±0.04 min) of the ES episodes was high. Cariporide, if applied alone, significantly decreased the incidence (0.03 ± 0.007 min^{-1}) and markedly reduced the average duration (0.066±0.05 min) of these episodes. The incidence and especially the duration of the ES episodes were also significantly reduced by both NCX inhibitors (ES_I: SEA: 0.05±0.02 min^{-1}; ORM: 0.05±0.02 min^{-1}; ES_D: SEA: 0.02±0.01 min; ORM: 0.02±0.007 min^{-1}). In contrast, if applied in combination with an NCX inhibitor, cariporide not only failed to improve, but virtually eliminated the beneficial effect of NCX inhibition on the duration and incidence of the ES episodes (SEA+CAR: 0.11±0.05 min and 0.09±0.03 min^{-1}; ORM+CAR: 0.17±0.09 min and 0.09±0.02 min^{-1}, respectively).

As in other arrhythmia types, in control hearts, both the incidence (0.17±0.04 min^{-1}) and average duration (0.14±0.05 min) of the periods with VT were high. Cariporide treatment provided the most effective anti-VT protection by dramatically decreasing both the incidence and duration of the post-ischaemic VT periods (VT_I: 0.01±0.002 min^{-1}; VT_D: 0.005±0.001 min). However, unlike the ES episodes, while both NCX inhibitors exerted a moderate beneficial effect, neither could significantly reduce the incidence or duration of the VT periods (SEA: 0.16±0.04 min^{-1} and 0.13±0.05 min; ORM: 0.10±0.03 min^{-1} and 0.09±0.07 min, respectively). Furthermore, the simultaneous application of cariporide and an NCX inhibitor resulted in the almost complete abolishment of the protection provided by cariporide treatment alone (SEA+CAR: 0.13±0.06 min^{-1} and 0.05±0.05 min; ORM+CAR: 0.09±0.07 min^{-1} and 0.10±0.05 min).

DISCUSSION

Failure of selective NCX inhibition to modulate ventricular AP

Selective NCX inhibition failed to modulate APD - neither as pretreatment nor following the exposure to ATX-II. The NCX inhibition induced uncoupling between I_{NaL} and $[Ca^{2+}]_i$ handling may also be effective under these conditions, however, it seems to exert only a minor effect on APD. Furthermore, considering the similar ineffectiveness of these inhibitors on APD we concluded that ORM-10103 should have negligible effect on increased APD dispersion. This observation contradicts to the results of Milberg et al. but supports the findings of Farkas et al. The reason of the discrepancy between our present findings and those of Milberg et al, is unclear. It can be different experimental condition, methods of

investigation, species differences. Importantly, the delicate nature of SEA0400 inhibition on I_{Ca} and NCX may play a role int he opposing resuts and needs further experiments.

The reason for the lack of effect on APD seems to be rather complex. Theoretically, the lengthening of APD following the enhancement of I_{NaL} may have two sources: (1) a *direct*, i.e. the effect of the increased I_{NaL} on APD, and (2) an indirect, i.e. the role of I_{NCX} in defining actual APD. We can assume that inhibition of NCX has no direct effect on I_{NaL} which may partially explain its failure to counteract APD prolongation. On the other hand, direct estimation of I_{NCX} kinetics during an AP is complicated following the application of ATX-II.

Inhibition of NCX, however, reduced $[Ca^{2+}]_i$ without an apparent effect on APD. It is possible that a primary reduction in CaT *via* negative feed-back prolongs ICa inactivation and subsequently lengthens APD. Therefore, if NCX inhibition had any direct effect on APD (i.e. I_{NCX} mediated abbreviation), it could be largely reduced by this indirect mechanism (i.e. I_{CaL} mediated prolongation). NCX inhibition, following the I_{NaL} induced rise in $[Ca^{2+}]_i$, may have two parallel, but opposite effects on APD: *directly,* it may abbreviate APD *via* inhibition of I_{NCX}, but *indirectly* - due to its reducing effect on $[Ca^{2+}]_i$ and the subsequent modulation of I_{Ca} kinetics - may also prolong it. Consequently, the actual balance of these two counteracting effects may intimately influence the overall result on APD of I_{NCX} inhibition, which is hard to predict, and may significantly differ in various arrhythmia models and species. Indeed, this complex relationship may explain the APD reduction by SEA0400, observed following a sotalol/veratridine challenge, and - under rather similar experimental conditions - the increased incidence of TdP in Langendorff perfused rabbit hearts following dofetilide treatment.

Global ischaemia

It is important to mention that the contractility increased in the SEA and the combinated groups compared to the control group. This can be explained by the SEA0400 inhibitory effect on NCX, which results in decreasing the Ca^{2+} removal from the cytosol, with a consequent increase in Ca^{2+} -transients and higher contractility.These results correspond with our previous work done on isolated rat cardiomyocytes and isolated heart under normoxia, where SEA0400 increased the Ca^{2+} transient amplitudes. However, since the SEA0400 inhibits both direction of the NCX, this observation can be explained only when Ca removal (forward NCX) is the dominant mode. NHE inhibition blocks the rise of intracellular Na^+, and therefore iindirectly prevents the Ca^{2+} overload through reverse NCX is. When applied in

combination with SEA0400, however, the reverse NCX is also inhibired directly by SEA0400. In this situation a synergistic effect could be expected by the combined NHE-NCX inhibition against Ca overload. Our results did not confirm this hypothesis, the exact reason for which is unknown. Since SEA0400 equally inhibits the reverse and forward mode NCX, we can speculate that, the attenuated Ca removal due to forward NCX block counteract the beneficial effect of the combined NHE- reverse NCX inhibition.

Regional ischaemia

The efficacy of two NCX inhibitors, the fairly selective SEA0400 and the novel, more selective ORM-10103, against reperfusion-induced arrhythmias were analysed in detail. Experimental data on the antiarrhythmic effects of SEA0400 are sparse and contradictory, although mostly positive, while similar data for ORM-10103 have become available only very recently. SEA0400 has a moderate inhibitory effect on the L-type Ca^{2+} channels, while ORM-10103 has a minor effect on I_{Kr}; neither of these pleiotropic effects could substantially modulate the observed antiarrhythmic efficacy of the NCX inhibition. Both inhibitors were applied alone or in combination with a well-characterised selective NHE inhibitor, cariporide. Cariporide is proposed to be highly selective and to not influence any of the important ion channels. It was assumed that the antiarrhythmic efficacy of a combination of NCX and an NHE inhibitor would be enhanced.

NHE inhibition (cariporide alone)

Our results obtained by the pre-ischaemic application of cariporide reflect the high antiarrhythmic efficacy of the treatment, and strongly support the conclusions of similar studies. Cariporide significantly increased the duration of the regular sinus rhythm and – with the exception of ES_D – *all arrhythmia-related variables* were significantly suppressed by the compound. The high antiarrhythmic efficacy is especially obvious in the case of VT and VF, since both arrhythmia types were almost completely eliminated following its application.

The background for the observed high efficacy of cariporide against VF and VT is not fully clarified. Data from previous studies indicate that during early reperfusion increased NHE activity promotes transient shortening of the APD, which renders the heart susceptible to *reentrant arrhythmias*, therefore NHE inhibition should significantly reduce the susceptibility of the heart to VF and VT. Furthermore, NHE inhibition has been shown to improve action potential (AP) propagation and reduce the ischaemia-induced increase in AP dispersion, most

probably via markedly delaying cell-to-cell electrical uncoupling. Since increased AP dispersion is a critical factor in the initiation and maintenance of VT and VF, improved AP conduction and subsequently decreased dispersion may be an important component in the strong anti-VT and -VF effect of cariporide.

NCX inhibition (SEA0400 or ORM-10103 alone)

The excellent protection against triggered arrhythmias may be due to strong inhibition of the *reverse NCX transport activity*, leading to a significant reduction in reperfusion-induced $Ca^{2+}{}_i$ load, while the lack of a similarly beneficial effect of SEA0400 on VFs and VTs suggests that to prevent the induction and maintenance of these arrhythmias, in addition to an effective reduction of the $Ca^{2+}{}_i$ overload, a similarly effective suppression of the re-entry activity is also essential.

In the present experimental model, VF had by far the largest contribution to total "arrhythmia time"; thus, its influence on the outcome of a pharmacological trial is predominant. Regarding its origin, VF is considered a re-entry type and not triggered-type arrhythmia; the key factor in its induction and maintenance is increased AP dispersion and ischaemia/reperfusion-induced spatial and/or temporal heterogeneities in the resting membrane potential. Consequently, since NCX inhibition is less suitable to protect the heart against *substrate-induced arrhythmias*, it is not surprising that the efficacy of SEA0400 and ORM-10103 against VF was also limited in our model. Furthermore, since ischaemia is short and incomplete in this arrhythmia model, the elevation of $[Ca^{2+}]_i$ during ischaemia and upon reperfusion is probably moderate, but heterogeneous. Therefore, activation of the reverse NCX transport mode is probably also moderate but heterogeneous, and the effect of NCX inhibition on the ischaemia/reperfusion-induced $[Ca^{2+}]_i$ overload in cardiomyocytes is uneven, unlike how it would be in the case of a severe, long-lasting ischaemia. Nonetheless, both NCX inhibitors substantially reduced the overall incidence of Ca^{2+}-dependent (triggered) arrhythmias, probably originating from locally injured regions. This finding is in line with most of the previous studies reporting the marked efficiency of NCX inhibition in reducing the formation of early and delayed after-depolarisations. Indeed, the significantly higher efficiency of NCX inhibition may be expected in conditions where the rise in $[Ca^{2+}]_i$ is substantially larger and the Ca^{2+} overload is more pronounced (e.g. following more extensive, complete or long-lasting ischaemia).

Combined NHE + NCX inhibition

While the results obtained with single inhibitors are logical and comprehensive, the outcome of the experiments with simultaneous NHE and NCX inhibition first seems highly surprising. Indeed, one would expect that the antiarrhythmic efficacy of simultaneous inhibition should exceed or, at least, meet the efficacy reached alone by any of the contributors. The fact that cariporide alone had significantly higher antiarrhythmic efficacy than in combination with an NCX blocker suggests that NCX inhibition was an important limiting factor in these groups. The reason for this apparent restrain is not clear at present and needs further investigation.

A logical, but largely hypothetical explanation may be as follows. In these experiments, the antiarrhythmic effect of cariporide could not be further improved by a synergic effect of NCX inhibition, since both NHE and reverse NCX inhibition could prevent the $[Na^+]_i$-induced rise in $[Ca^{2+}]_i$. Indeed, this assumption is supported by the almost identical effects of combined NCX+CAR and NCX alone inhibition. The decreased efficiency of cariporide is most probably caused by *inactivation* of its beneficial effect on the arrhythmogenic substrate.

The observed "loss of effect" of cariporide may be related to the significant inhibition of the forward transport mode of NCX by SEA0400 or ORM-10103. Indeed, one may speculate that the beneficial anti-VF effect of cariporide (applied alone) should manifest in decreasing the spatial and/or temporal heterogeneities below the critical level required to evoke fibrillation. Simultaneous inhibition of the forward transport mode of NCX may exert an opposite effect by increasing these heterogeneities via inhibiting Ca^{2+} extrusion needed for restoration of the normal Ca^{2+} cycle of the cell. This effect may be less important in cardiomyocytes affected by a lower level of ischaemia and subsequent $[Ca^{2+}]_i$ load compared to those with more significant rise in $[Ca^{2+}]_i$. On the other hand, NHE inhibition may remain latent in normal, non-ischaemic myocardium, since NHE is mostly inactive at normal pH_i, while NCX inhibition undoubtedly affects Ca^{2+} handling and AP kinetics probably differentially in the ischaemic and non-ischaemic myocardium.

CONCLUSION

Our present data support the hypothesis that selective, partial NCX inhibition may be antiarrhythmic via restricting the Na^+-induced $[Ca^{2+}]_i$ elevation, and this protective effect is mediated primarily by its inhibitory effect on $_{rev}I_{NCX}$. Therefore, NCX inhibition can be considered as a promising therapeutic strategy against Ca^{2+} overload-induced, $_{rev}$NCX-mediated cardiac arrhythmias.

In principle, inhibition of either the NCX or the NHE and especially their *combined* inhibition should effectively protect the heart against reperfusion-induced harmful arrhythmias, since both mechanisms target and block the same $[Ca^{2+}]_i$ overload pathway, at different points. The results of this study, however, are much less straightforward and do not fully support this presumption. Therefore, we concluded that NCX inhibition alone, or in combination with NHE blockade, appears to be a less than optimal therapeutic strategy against the full range of reperfusion-induced arrhythmias. A possible reason for the limited efficacy may be that the currently available selective NCX inhibitors with their equally effective and long-lasting bidirectional blockade of the NCX activity are not fully suitable for this purpose. On the other hand, these NCX inhibitors proved to be highly effective against EAD- and DAD-induced arrhythmias. To better explore the suggested therapeutic potential of combined NCX+NHE inhibition, further studies based on novel NCX inhibitors with different inhibitory characteristics are needed.

ACKNOLDEGEMENTS

I am very grateful to Professor András Varró, MD, DSc, for his continuous support and for providing me the opportunity for research at the Department of Pharmacology and Pharmacotherapy and to Professor Gyula Papp MD, DSc, academician for his criticism and helpful advice.

I am especially thankful to my supervisors András Tóth, PhD, for his continuous support and personal guidance at the Department of Pharmacology and Pharmacotherapy and for introducing me to the techniques. His personal guidance and helpful discussions were useful during my work and allowed me to learn the critical thinking in the scientific field

I wish to thank my grateful colleague, Károly Acsai PhD, for his continuous support and help to study the experimental techniques in the field of the electrophysiology.

I would like to thank all my direct colleagues, Norbert Nagy PhD; János Prorok PhD, Anita Kormos PhD; Andrea Gruber for their help in my work. I am also very thankful to Zsuzsanna Sebők and Imréné Molnár for her helpful technical assistance.

A particular acknowledgement goes to my parents, brother, sister, and all my friends for their help and encouragement, to whom I dedicated this PhD thesis. Finally, my deepest appreciation goes to my love, Sándor, for his love, support and encouragement in the past years.

The publication is supported by the **Richter Gedeon Centenáriumi Foundation.**