Investigation of the cardioprotective effect of selective NCX inhibition in cellular models

PhD Thesis

Anita Kormos, MSc

Department of Pharmacology and Pharmacotherapy

University of Szeged

Szeged

Hungary

2015

STUDIES RELATED TO THE THESIS

I. *Kormos A, Nagy N, Acsai K, Vaczi K, Agoston S, Pollesello P, Levijoki J, Szentandrassy N, Papp JGy, Varro A, Toth A*
Efficacy of selective NCX inhibition by ORM-10103 during simulated ischemia/reperfusion.
EUROPEAN JOURNAL OF PHARMACOLOGY 740: pp. 539-551(2014)
IF.: 2,754

II. *Nagy N, Kormos A, Kohajda Z, Szebeni A, Szepesi J, Pollesello P, Levijoki J, Acsai K, Virag L, Nanasi PP, Papp JGy, Varro A, Toth A*
Selective Na^+/Ca^{2+} exchanger inhibition prevents Ca^{2+} overload induced triggered arrhythmias.
BRITISH JOURNAL OF PHARMACOLOGY 171:pp.5665-5681(2014)
IF.: 4,99

OTHER STUDIES

I. *Nagy N, Acsai K, Kormos A, Sebők Zs, Farkas AS, Jost N, Nánási PP, Papp JGy, Varró A, Tóth A*
$[Ca^{2+}]_i$-induced augmentation of the inward rectifier potassium current (I_{K1}) in canine and human ventricular myocardium.
PFLÜGERS ARCHIV - EUROPEAN JOURNAL OF PHYSIOLOGY 465:(11) pp. 1621-1635 (2013)
IF.: 3,073

ABSTRACTS

I. *Prorok J., Nagy N., Kormos A., Acsai K., Papp J.Gy., Varró A., Tóth A.*
A Na^+/Ca^{2+} cseremechanizmust blokkoló SEA0400 hatása intracelluláris szívizomsejtekben.
CARDIOLOGIA HUNGARICA 38:Suppl.B: p.B20 (2008)

II. *Nagy N, Szentandrássy N, Szebeni Á, Kormos A, Acsai K, Nánási P, Papp JGy, Varró A, Tóth A*
Inhibition of sodium-calcium exchanger reduces the sodium induced calcium overload in canine myocardium. (A nátrium-kálcium exchanger gátlás csökkenti a nátrium indukált calcium overloadot kutya szívizomban)
CARDIOLOGIA HUNGARICA 41:(Suppl. F) pp. F38-F39. (2011)

III. *Kormos A, Nagy N, Szebeni A, Szentandrassy N, Acsai K, Papp JGy, Varró A, Tóth A*
Partial NCX inhibition - via limiting Ca^{2+} influx - exerts a protective role against Na^{+}-induced Ca^{2+} overload in canine ventricular myocardium. (Részleges NCX-gátlás - a Ca^{2+} -beáramlás csökkentésével - védő hatást fejt ki a Na^{+}-indukált Ca^{2+}-túltöltődés ellen kutya kamrai szívizomban.)
CARDIOLOGIA HUNGARICA 42:(Suppl.A) p. A18. (2012)

IV. *Kormos A, Márton Z, Oravecz K, Jost N, Varró A, Papp JGy, Acsai K*
A szelektív Na^{+}/Ca^{2+} exchanger (NCX) gátlás inotrop hatása a Ca^{2+} -influx és -efflux mód Ca^{2+}-egyensúlyban betöltött relatív szerepétől függ kutya szívizomsejtekben. (Inotropic consequences of selective Na^{+}/Ca^{2+} exchange inhibition depend on the relative contribution of Ca^{2+} influx and efflux mode to the Ca^{2+} balance of dog cardiac myocytes.)
CARDIOLOGIA HUNGARICA 43:(Suppl.B) pp. B21-B22. (2013)

V. *Oravecz K, Kormos A, Acsai K*
A nátrium-kalcium exchanger új gátlószereinek vizsgálata a szívelégtelenség kezelésében kedvező pozitív inotróp és antiaritmiás hatások tekintetében
In:
„Hiteles(ebb) tudományos prezentációk" című VIII. Ph.D. - Konferencia előadásai. Konferencia helye, ideje: Budapest, 2014.03.13. II. Kötet. pp. 23-25.(2014)

VI. *Oravecz K, Kormos A, Acsai K*
Investigation of the possible positive inotropic and antiarrhythmic efficacy of ORM 10103, a new selective inhibitor of the cardiac sodium-calcium exchanger
In: Szélpál Sz (szerk.)
I. Innovation in Science – Doctoral Student Conference Konferencia helye, ideje: Szeged, 2014.05.02-2014.05.03. eBook of Abstracts: p. 23. (2014)

INTRODUCTION

Intracellular Ca^{2+} and Na^{+} homeostasis

Intracellular Ca^{2+} cycling in the cardiomyocytes is a result of the tightly regulated Ca-influx and efflux pathways. The membrane depolarization initiates the sarcolemmal Ca^{2+} influx through the L-type Ca^{2+} channels, which are located in the membrane of T-tubules near the junctional region of the sarcoplasmic reticulum (SR). This "trigger calcium" is thought to directly activate the calcium release channels embedded in the junctional SR. Opening of these calcium-sensitive Ca^{2+} release channels (ryanodine receptors, RyR) partially empties the internal store of calcium. This mechanism is known as calcium-induced Ca^{2+} release (CICR).

Binding of calcium to troponin C in the contractile apparatus initiates muscle contraction (systole). Reuptake of calcium into the sarcoplasmic reticulum by the phospholamban-regulated sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) allows for cardiac relaxation (diastole). β receptor–mediated protein kinase A (PKA) stimulation regulates this Ca^{2+} cycling by phosphorylating L-type Ca^{2+} channels, RyR, and phospholamban. In normal hearts, sympathetic stimulation activates β_1-adrenergic receptor, which in turn stimulates the production of cAMP and thereby activates PKA. PKA phosphorylates phospholamban and RyR, both of which contribute to an increased intracellular $[Ca^{2+}]_i$ transient. Beside to these channels, NCX plays an essential role in removal of Ca^{2+}, also contributes to the Ca^{2+} trigger. The cardiac contractility and force development depend on the free intracellular Ca^{2+} level and the Ca^{2+} sensitivity of the cardiomyocytes. SERCA and the NCX play key roles in maintaining the balance of the cellular Ca^{2+} homeostasis.

In steady-state conditions intracellular Na^{+} is determined by the balance between Na^{+} influx and efflux. There are four major pathways for Na^{+} influx: 1. Voltage-gate Na^{+} channels, 2. Na^{+}/H^{+} exchange (NHE), 3. Na^{+}- HCO_3^{-} cotransporter (NBC), 4. Na^{+}/Ca^{2+} exchange. Intracellular Na^{+} concentration and Na^{+} pump activity belong to the most important factors of the NCX regulation. Indeed, in experimental models of heart diseases associated with increased internal Na level (for example in ischemia/reperfusion injury), activity of NCX can be significantly elevated, which can contribute to the cellular the $[Ca^{2+}]_i$ overload.

The sarcolemmal Na^+/K^+ ATP-ase activity is essential for the Na^+ gradient that drives ion transport processes critical for normal cardiac function. This pump transports 3 Na^+ and 2 K^+ against their electromechanical gradients for each ATP hydrolysed, maintaining the low intracellular Na^+ level and the high K^+ level. That is the reason why the Na^+/K^+ ATP-ase is very important in the cardiac myocytes. The transsarcolemmal Na^+ gradient established by the Na^+ pump activity is important for AP depolarization and regulation of NCX.

Effect of ischemia/reperfusion in cardiomyocytes

Myocardial ischemia has a major consequence: it is acidosis and this contributes to the ischemic decline in force, in large part for decreased myofilament Ca^{2+} sensitivity. $[Ca^{2+}]_i$ transient amplitude can be initially decreased. Ischemia can also increase diastolic $[Ca^{2+}]_i$. Low intracellular pH stimulates proton extrusion via NHE (and NBC), that is the reason why Na^+ influx is increased. Furthermore, the Na^+/K^+ pump is partially inhibited at low pH. These factors lead to the slow recovery of contractility via shift in NCX and increase in $[Ca^{2+}]_i$ and could also lead to $[Ca^{2+}]_i$ overload and arrhythmias. $[Ca^{2+}]_i$ overload can lead to cell necrosis during the reperfusion period. Increase in $[Ca^{2+}]_i$ and oxidative stress-induced dysfunction of sarcoplasmic reticulum are the reasons for cell damage during reperfusion. These also contribute to the pathogenesis of ischemia/reperfusion induced injury. During ischemia the major way of Ca^{2+} entry is reverse mode NCX, due to sarcolemmal depolarization and increased intracellular Na^+ concentration. In fact, decreased ATP phosphorylation potential blocks the activity of the sarcolemmal Na^+/K^+ ATPase, the intracellular Na^+ concentration rises and the sarcolemma is being depolarized. Cytosolic $[Na^+]$ overload is accentuated by intracellular acidosis, which induces Na^+ entry through the NHE. The ischemia associated acidosis contributes to increase cytosolic Ca^{2+} induced by the NHE, which is strongly coupled to NCX, so that in long term the cytosolic H^+ is exchanged with extracellular Ca^{2+}. Inhibitors of the Na^+/H^+ exchanger were found to be protective if used before ischemia, while their effectiveness was reduced when given during the reperfusion phase.

The future perspectives of NCX modulation

Since pharmacological inhibition of the NCX exchanger proved to be beneficial in experimental models of cardiac disorders, selective NCX inhibitors may represent a novel group for the treatment of cardiac diseases. For example, intracellular accumulation of Na^+ in the myocardium during ischemia/reperfusion favors the reverse mode operation of NCX, which may contribute to the intracellular accumulation of Ca^{2+} leading to cell damage and death. Therefore, pharmacological inhibition of NCX by ORM -10103 may provide cardioprotection by restricting excessive Ca^{2+} accumulation via inhibition of the reverse mode operation of NCX.

Prolongation of the QT interval is a severe risk factor in a number of cardiovascular diseases. Two of the genes responsible for long QT syndrome (LQTS), have been identified as *KCNH2* (LQT2) and *SCN5A* (LQT3). ATX-II is an inhibitor of sodium-channel inactivation that thereby mimics LQT3, in which a mutation in *SCN5A* leads to a small, persistent component of the inward, depolarizing ion current (I_{Na}) via continuous reopening of the sodium channel. I_{Na} is responsible for rapid initial action potential depolarization. Furthermore abnormalities in I_{Na} inactivation produce large inward Na^+ currents during the cardiac action potential plateau, causing arrhythmias. Against increased I_{NaL} induced pathologic alterations in $[Ca^{2+}]_i$ homeostasis selective NCX inhibition by ORM-10103 could be highly protective.

The major problem, however, with the currently available NCX inhibitors is the lack of their selectivity. Concomitant inhibition of the L-type Ca^{2+} current is the most important limitation of their use in experimental studies. Although combined block of I_{CaL} and I_{NCX} can also be useful in certain clinical settings, it makes difficult to interpret the beneficial effects of the pure NCX inhibition. Therefore, experimental use of the newly developed, selective NCX inhibitors is a major advancement in the field of the NCX research.

Aims of the study

The principal aim of the study was to directly study how partial inhibition of the Na^+/Ca^{2+} exchanger via the novel, selective pharmacological agent ORM-10103 influences the elements of Ca handling in cardiac cells. Our results describe the effect of the application ORM -10103 in dog ventricular cells under increased intracellular Ca^{2+} level.

The aims of this study were:

- To investigate the protective effect of a novel, selective NCX inhibitor ORM-10103 against the adverse effects of pharmacologically induced Na^+-induced Ca^{2+} load.
- To establish a cellular level ischemia/reperfusion injury model (moderate and severe ischemia), in which we can characterize the survival rate of the cells, as well as the major electrophysiological parameters of AP and CaT.
- To estimate the potential therapeutic possibilities of selective NCX inhibition against adverse effects of ischemia reperfusion injury, applying ORM-10103.

RESULTS

The physiological effect of selective NCX inhibition on the $[Ca^{2+}]_i$ transient

Cardiomyocytes were loaded with a Ca^{2+}-sensitive fluorescent dye, Fluo 4-AM. In time control measurements only a small, gradient decrease in the magnitude and baseline of the transient could be observed. This gradual decrease is predominantly a consequence of dye leakage/extrusion from the cell. Interestingly, in normoxic conditions the application of 10 μM ORM-10103 had no apparent effect on the magnitude or kinetics of the transient.

Effect of selective NCX inhibition on the I_{NaL} induced increase in $[Ca^{2+}]_i$ transient

I_{NaL} was activated by 2 nM ATX-II which is known to increase substantially the Na^+ influx due to lengthening of the inactivation of I_{NaL}. Application of 2 nM ATX-II significantly enhanced the magnitude of the $[Ca^{2+}]_i$ transient, but this increase was diminished by subsequent application of 10 μM ORM10103.

ORM-10103 decreased the spontaneous diastolic Ca^{2+} releases evoked by Na^+/K^+ pump inhibition

We hypothesized that ATX-II induced EADs are caused by Ca^{2+} release from SR (which also causes DADs), rather than by reactivation of the L-type Ca^{2+} current during prolonged AP. The pacing frequency was set to 1 Hz. During NKA inhibition via 1 μM strophantidine resulted in significantly enhanced $[Ca^{2+}]_i$ transient. Pretreatment with 10 μM ORM10103 markedly reduced the effect of the subsequently applied strophantidine. This 1 Hz period was than followed by the period of 2 Hz stimulation and a third period with stopped stimulation. Since during the 2 Hz period a substantial number of large diastolic releases could be observed, it seems feasible that under these experimental conditions a substantial $[Ca^{2+}]_i$ overload was generated. In the absence of strophantidine (control) spontaneous diastolic Ca^{2+} release was not observed following a short period of rapid (2 Hz) pacing. In contrast, rapid pacing induced multiple arrhythmogenic Ca^{2+} release events (presumably resulting in DADs) in the presence of 1 μM strophantidine. Following 10 μM ORM10103 pretreatment the same strophantidine challenge was much less effective to evoke spontaneous Ca^{2+} release.

3.4. Effect of selective NCX inhibition on cardiomyocyte viability under ischemic conditions

Instead of using vital dyes, we followed a similarly effective classification, based simply on the shape and visibility of the striation of the cell. Based on their morphological characteristics cells in the ROI were classified into two groups: Class A: elongated cells with intact border and clearly visible striation. Class B: dead cells, or cells on the verge of death with no visible striation and typically in full contracture.

Mean values, determined for both groups in the last minute of the control (normoxic), ischemia and reperfusion periods, are summarized in panel. The distribution of cells in the normoxic state was close to identical in both groups, and the majority of these cells were intact (78% and 81% in the untreated and ORM-10103 treated groups, respectively). As noted above, ischemia by itself apparently did not influence cell distribution in either group. In contrast, reperfusion had a detrimental effect on the untreated group: 71% (112 out of 156) of the cells intact at the end of ischemia died during reperfusion. Application of 10 μM ORM-10103 had a clearly protective effect on cell viability; significantly less, only 47% (76 out of 161) of the ORM-treated cells died by the end of the reperfusion period.

The effect of selective NCX inhibition on the $[Ca^{2+}]_i$ transient during simulated ischemia

During simulated ischemia in both strophantidine untreated and treated cells significant changes in the magnitude and kinetics of the CaT could be observed. Significant changes in the amplitude of the CaT in untreated compared to time control cells could only be observed during the early phase of ischemia (0.836 ± 0.03 vs. 0.979 ± 0.02; n=14) (B). In contrast, 10 μM ORM-10103 caused a steady, gradual decrease in the amplitude of the CaT (0.76 ± 0.06 vs. 0.38 ± 0.05; n=14), which became significant (compared to ORM-10103 untreated cells) during reperfusion. The largest modulatory effect of ORM-10103 treatment could be observed in $[Ca^{2+}]_{iD}$. As expected, ischemia induced a substantial rise in $[Ca^{2+}]_{iD}$, which became significant by the 2nd min of ischemia. Upon reperfusion $[Ca^{2+}]_{iD}$ slowly normalized, however, during its early phase (~6 min) it was still significantly higher than in time control cells (1.04 ± 0.03 *vs.* 0.92 ± 0.02; n=14). Pretreatment of the cardiomyocytes with 10 μM ORM-10103 completely eliminated the rise in $[Ca^{2+}]_{iD}$ and by the end of the ischemic period it even decreased below time control

(0.73 ± 0.08 *vs.* 0.64 ± 0.06; n=14). During reperfusion $[Ca^{2+}]_{iD}$ in these cells was permanently below control.

Ischemia/reperfusion induced changes in characteristic parameters of the CaT in *strophantidine treated* cells. In these cells no significant changes in the amplitude of the CaT could be observed during ischemia or reperfusion. Compared to time control, the slope of the CaT gradually, but significantly decreased during both ischemia and reperfusion. ORM-10103 failed to influence the ischemia induced fall in the slope of the CaT, but significantly limited its further decrease during reperfusion (0.55 ± 0.1 vs. 0.21 ± 0.02; n=6). In ORM-10103 untreated cells RT_{50} was close to the time control during both ischemia and reperfusion. In contrast, the application of 10 μM ORM-10103 induced a significant decrease in this parameter during reperfusion (1.17 ± 0.11 vs. 0.81 ± 0.06; n=6). The already significant ischemia-induced elevation in $[Ca^{2+}]_{iD}$, observed in the untreated myocytes, was further significantly augmented in strophantidine treated cells and this marked increase was apparently maintained during the entire period of reperfusion. Application of 10 μM ORM-10103 completely eliminated the huge ischemia induced elevation in $[Ca^{2+}]_{iD}$.

The beneficial effect of ORM-10103 treatment during reperfusion became even more evident by comparing short term variabilities of the CaT amplitudes. Representative Poincare plots, obtained in untreated, ORM-10103 treated, strophantidine treated and strophantidine + ORM-10103 treated cells. In untreated cells no apparent ischemia/reperfusion induced changes in CaT variabilities could be observed, while ORM-10103 treatment caused a small, rather insignificant decrease in both cases during ischemia and reperfusion, respectively. In contrast, as shown in panel, during ischemia strophantidine significantly enhanced the short term variability which was even further augmented during reperfusion. This large elevation was, again, fully eliminated by the application of 10 μM ORM-10103.

Effect of selective NCX inhibition on AP parameters

During normoxia 10 μM ORM-10103 evoked moderate APD shortening without substantially modulating either its amplitude or the resting membrane potential. During simulated ischemia, however, significant changes in the shape and kinetics of the APs

developed and the resting membrane potential was depolarized. Both parameters were apparently normalized during reperfusion. The AP shortening effect of 10 μM ORM-10103 observed in normoxic cells was even more evident during both ischemia and reperfusion.

In order to characterize major changes in AP kinetics APD_{25} and APD_{90} were determined and from these variables AP triangulation was calculated. Ischemia induced a moderate decrease in APD_{25} which was normalized during reperfusion. Compared to the untreated cells substantially larger decrease in APD_{25} could be observed in ORM-10103 treated cardiomyocytes, became significant in the late phase of ischemia and did not recover during reperfusion (0.90 ± 0.09 *vs.* 0.66 ± 0.09; n=9). Qualitatively similar, but augmented ischemia induced shortening could be observed in APD_{90}, however, the differences between the two groups were not significant during either ischemia or reperfusion. Compared to the time control cells, ischemia induced a large, significant decrease in AP triangulation in both groups, but again, the differences between the untreated and ORM-10103 treated groups were not significant (0.61 ± 0.07 *vs.* 0.76 ± 0.17; n=9).

Under normoxic conditions APD variabilities were similar in both groups. Ischemia induced moderate, but insignificant decrease in APD_{90} variabilities in the untreated group, while there was no apparent change in the ORM-10103 treated group. In contrast, APD_{25} variabilities failed to change in the control group, but showed a tendency to decrease following ORM-10103 treatment. During reperfusion the only significant change was an elevation in APD_{90} variabilities determined in the untreated group. ORM-10103 treatment eliminated this increase (5.37 ± 0.96 *vs.* 3.47 ± 0.69 ms; n=9).

DISCUSSION

The possible antiarrhythmic effect of NCX inhibition can be attributed to its important role to prevent the I_{NaL}-mediated and ischemia/reperfusion induced $[Ca^{2+}]_i$ overload. In both pathological conditions partial NCX inhibition may restore the balance in intracellular Ca^{2+} handling, resulting in decreased propensity for arrhythmias. Since the cardiac Na^+/Ca^{2+} exchanger has a crucial role in maintaining $[Ca^{2+}]_i$ homeostasis, any abnormal shift in its transport rate may significantly contribute to alteration of the contractile function and electric activity of the heart. In our experiments the effects of

selective I_{NCX} inhibition on $[Ca^{2+}]_i$ homeostasis have been evaluated under arrhythmogenic conditions.

Enhanced reverse mode activity may induce $[Ca^{2+}]_i$ overload, while its enhanced forward mode transport leads to gradual $[Ca^{2+}]_i$ loss. While a number of previous studies provided important information on the consequences of NCX inhibition in healthy and diseased hearts, interpretation of these results was hampered by the fact, that the applied inhibitors were not selective. In this respect, our results allow new insight into the physiological and pathophysiological role of NCX, since we used ORM-10103, a new NCX inhibitor with excellent specificity and selectivity during Na^+ induced $[Ca^{2+}]_i$ load and during simulated ischemia/reperfusion in isolated canine ventricular cardiomyocytes. The most important effects of ORM-10103 were studied on CaT and AP, parameters relevant in terms of arrhythmia development. Application of 10 μM ORM-10103 had a clearly protective effect on cell viability because numerous of ORM-untreated cells died by the end of the reperfusion period. Moreover ischemia induced a substantial rise in $[Ca^{2+}]_{iD}$, and the application of 10 μM ORM-10103 completely eliminated this rise in $[Ca^{2+}]_{iD.}$ Furthermore, during ischemia/reperfusion the short term variability of the CaT and AP - a well known arrhythmia marker – became significantly enhanced in strophantidine treated cells, and this large increase was fully eliminated by the application of 10 μM ORM-10103.

Selective NCX inhibition does not influence CaT kinetics under physiological conditions

Under physiological conditions we did not find apparent effect on CaT parameters in the present of NCX inhibition by ORM-10103. These results are in line with our previous work. The background of this effect is complex. As we can find it in the literature, markedly different results were obtained in small species, where NCX inhibition increased the magnitude of $[Ca^{2+}]_i$ transients. This may be due to the short action potential duration in contrast to dogs and humans, where the action potential duration is longer. NCX spends relatively longer time in its forward mode during the AP in small species (rat) than in larger animals (dog), which may lead to increased $[Ca^{2+}]_i$ transient when NCX is blocked. This is the possible reason why in our canine myocytes ORM-10103 induced changes in CaT was failed.

The effect of selective NCX inhibition against I_{NaL} induced $[Ca^{2+}]_i$ rise on the $[Ca^{2+}]_i$ parameters

The selective NCX inhibitor ORM-10103 effectively reduced the increased $[Ca^{2+}]_i$ and prevented the I_{NaL}-mediated rise in $[Ca^{2+}]_i$. The ATX-II induced I_{Na} increase leads to net gain in $[Na^+]_i$, shifting subsequently the reversal potential of the NCX in ventricular cardiomyocytes. The I_{NaL} activation caused increase in the $[Ca^{2+}]_i$, which was prevented following the selective NCX blockade. This secondary increase in $_{rev}I_{NCX}$ was fully abolished by pretreatment with 10 μM ORM-10103. This clearly indicates the pathogenetic role of reverse NCX in the I_{NaL} -induced $[Ca^{2+}]_i$ overload and arrhythmia. Thus, these data support important role in cardiac arrhythmogenesis of the I_{NaL} activation and may suggest the beneficial antiarrhythmic effects of NCX inhibition.

Selective NCX inhibition increased the cell survival during ischemia/reperfusion

A most direct way to test the efficacy of the selective NCX blockade in protecting cardiomyocytes against ischemia/reperfusion injuries is to compare cell survival between untreated and treated groups. The results of these experiments are straightforward and seem to support the hypothesis that partial, selective NCX inhibition by ORM-10103 may effectively protect the cardiomyocytes from severe ischemia/reperfusion induced injuries. This protection is most probably a direct consequence of the NCX inhibitory effect on the buildup of ischemia-induced $[Ca^{2+}]_i$ overload leading to contracture and elevated $[Ca^{2+}]_{iD}$, which may severely compromise mitochondrial function. A further contribution of ORM-10103 to cardiomyocyte survival may be its significant stabilizing effect on the CaT and APD by inhibiting the ischemic increase in their variabilities.

The effect of selective NCX inhibition during simulated ischemia/reperfusion on the $[Ca^{2+}]_i$ parameters

According to our results consequences of $[Ca^{2+}]_i$ accumulation could be observed in untreated cells. Diastolic $[Ca^{2+}]_i$ significantly increased and since the amplitude of the transient did not decrease simultaneously, systolic $[Ca^{2+}]_i$ was also enhanced. The kinetic parameters of CaT were also altered. The slope of the transient decreased, while RT_{50} increased during ischemia, reflecting the relative energy deficit as a consequence of the metabolic transition. The background of this effect may be rather complex: ATP depletion reduces SERCA activity (leading to decreased Ca^{2+} content of the SR; see the reduced

slope and amplitude of the CaT), the increased resting membrane potential decreases the drive for the forward, the high $[Na^+]_i$ increases the drive for reverse NCX current, the latter facilitates Ca^{2+} influx leading to elevated $[Ca^{2+}]_i$ level to be extruded. Therefore, we suggest that in this experimental model the $Ca^{2+}{}_i$ content of the SR is decreased (via reduced SERCA and forward NCX activity), the $[Ca^{2+}]_i$ level is elevated. Upon reperfusion the amplitude and slope of the $[Ca^{2+}]_i$ transient and – although quite slowly – the diastolic $[Ca^{2+}]_i$ were restored. In contrast, RT_{50} was significantly shortened during reperfusion. The acceleration of $[Ca^{2+}]_i$ transient relaxation in reperfusion may be related to quick recovery of normal K^+ level, and membrane hyperpolarization, which may significantly increase the speed and efficacy of forward NCX.

Pretreatment with 10 μM ORM-10103 displayed an important modulatory effect on the response of cardiomyocytes to ischemia/reperfusion. More importantly, the ischemia induced large increase in diastolic $[Ca^{2+}]_i$ and the elevation of RT_{50} were completely blocked by ORM-10103 treatment and in this case a significant decrease in these parameters could be observed during reperfusion. These results provide direct support for the hypothesis that during ischemia/reperfusion the predominant effect of ORM-10103 treatment is an effective suppression of the $[Na^+]_i$ accumulation induced massive activation of the reverse mode activity of NCX.

A further support to this conclusion was provided by the results from the strophantidine experiments. Application of 1 μM strophantidine blocks the sarcolemmal Na^+/K^+-ATP-ase effectively, thus facilitating intracellular Na^+ accumulation, which in turn, shifts NCX activity to even more reverse direction. The ischemia induced elevation of diastolic $[Ca^{2+}]_i$ in these cells was higher than in cardiomyocytes not treated with strophantidine, and in contrast to the untreated cells, it was further increased during reperfusion. However, this effect was completely blocked by 10 μM ORM-10103.

The results of the CaT experiments seem to strongly support the beneficial effects of ORM-10103 treatment on the $[Ca^{2+}]_i$ homeostasis in cardiomyocytes during I_{NaL}-mediated rise in $[Ca^{2+}]_i$ and an ischemia/reperfusion injury, by practically eliminating the $[Ca^{2+}]_i$ overload together with its detrimental consequences and by largely decreasing the arrhythmia propensity of the heart.

Selective NCX inhibition causes only minor changes on AP morphology

The reason for this unability of the NCX inhibitor to prevent the protective effects of ischemia/reperfusion on the AP may be quite complex. Changes in $[Ca^{2+}]_i$ are likely important modulators of the membrane potential at any time during the cardiac cycle however, the shape and kinetics of the AP are much more dependent on several other parameters, including the inward Na^+ and outward K^+ currents. Neither of these were affected directly by ORM-10103. This may be the primary cause for the limited sensitivity of the AP to selective NCX blockade. The only apparent beneficial effect of ORM-10103 on variables of the action potential was a significant reduction in reperfusion-induced rise of APD_{90} variabilities.

CONCLUSION

Until recently, the lack of proper NCX inhibitors significantly hampered the efforts to answer the question, how selective NCX inhibition would modulate the arrythmogenic consequences of severe Ca^{2+} overload, induced by either increased late sodium current or ischemia/reperfusion. That is the reason, why the overall conclusion of the present study, i.e. that treatment by a highly selective NCX inhibitor, ORM-10103 proved to effectively improve the cell viability during ischemia/reperfusion injury, seems to be really important.

ORM-10103 apparently normalized the stability of the $[Ca^{2+}]_i$ transient markedly weakened during reperfusion and facilitated the post-reperfusion survival of the cardiomyocytes. Its beneficial, preventive action against the arrhythmogenic shifts in $[Ca^{2+}]_i$ homeostasis caused by elevated late sodium current or ischemia/reperfusion can apparently be a consequence of its limiting effect on $[Ca^{2+}]_i$ overload and subsequent elimination of the ischemia-induced rise in diastolic $[Ca^{2+}]_i$. These effects may have primarily contributed to the effective inhibition of the $[Na^+]_i$ rise-induced, significantly enhanced reverse mode transport activity of the Na^+/Ca^{2+} exchanger. On the other hand, in certain pathological conditions, where the source of arrhythmia is the disturbed AP morphology and to lesser extent the $[Ca^{2+}]_i$ overload, the protective efficacy of ORM-10103 is rather limited, since it is apparent unable to prevent or significantly decrease these arrhythmogenic AP changes. Therefore, it can be stated that selective NCX inhibition might best be used to protect the heart against the arrhythmogenic effects of Na^+ induced $[Ca^{2+}]_i$ overload and the subsequent cell death.

Selective NCX inhibitors might be the key to find a way to have antiarrhythmic effect against the I_{NaL}-mediated and ischemia/reperfusion induced $[Ca^{2+}]_i$ overload and cell death.

ACKNOWLEDGEMENTS

I am especially thankful to my supervisors **András Tóth, PhD**, for introducing me to the cellular fluorescent techniques and conventional microelectrode techniques, and for providing me the opportunity to work in the optical laboratory, and to **Professor András Varró, MD, DSc,** for his permanent support during my work at the Department of Pharmacology and Pharmacotherapy.

I am very grateful to **Professor Julius Gy. Papp, MD, DSc, member of the Hungarian Academy of Sciences**, for his permanent support, personal guidance, criticism, and suggestions. The helpful discussions were exceptionally useful during my work and improved my critical thinking that is needed in the scientific field.

I wish to thank my grateful colleagues, **Norbert Nagy PhD**, and **Károly Acsai PhD**, for their continuous support during my work in the field of the electrophysiology.

I would like to thank all my direct colleagues, **Judit Szepesi MSc**, **Nikolett Nagy MSc**, and to **János Prorok PhD** for their help in my work. I am also very thankful to Ms. **Andrea Gruber MSc** for her helpful technical assistance.

Finally, I wish to thank, and dedicate this thesis to my whole family and to my friends for their love, help and encouragement. Last but not least, my deepest appreciation goes to my husband, for his love, support and encouragement.

The publication is supported by the Richter Gedeon Talentum Foundation and by grants from the Hungarian Scientific Research Fund (NK-104331), the National Office for Research and Technology-Baross Programmes (REG-DA-09-2-2009-0115-NCXINHIB), the National Development Agency and co-financed by the European Regional Fund (TÁMOP-4.2.2A-11/1/KONV-2012-0073 and TÁMOP-4.2.2.A-11/1/KONV-2012-0060), the HU-RO Cross-Border Cooperation Programmes (HURO/1001/086/2.2.1_HURO-TWIN) and the Hungarian Academy of Sciences.