EXPERIMENTAL INVESTIGATION OF THE ACTION POTENTIAL DURATION INFLUENCING ANTIARRHYTHMIC DRUGS IN VIVO

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PhD Thesis

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PUBLICATIONS RELATED TO THE THESIS

I. Long-term pretreatment with desethylamiodarone (DEA) or amiodarone (AMIO) protects against coronary artery occlusion induced ventricular arrhythmias in conscious rats.

Morvay N, Baczkó I, Sztojkov-Ivanov A, Falkay G, Papp JG, Varró A, Leprán I.
Canadian Journal of Physiology and Pharmacology 93 (9):773-7 (2015)

II. The effect of a novel highly selective inhibitor of the sodium/calcium exchanger (NCX) on cardiac arrhythmias in *in vitro* and *in vivo* experiments.

Kohajda Z, **Farkas-Morvay N**, Jost N, Nagy N, Geramipour A, Horváth A, Varga RS, Hornyik T, Corici C, Acsai K, Horváth B, Prorok J, Ördög B, Déri S, Tóth D, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Baczkó I, Leprán I, Nánási PP, Papp JG, Varró A, Virág L.
PLoS One. 2016 Nov 10;11(11):e0166041.

QUOTABLE ABSTRACTS RELATED TO THE THESIS

I. Augmented reverse mode of Na^+/Ca^{2+} exchanger may contribute to the preserved pump function in experimental heart failure

Kohajda Zs; Szlovák J; Gazdag P; **Morvay N**; Prorok J; Szépe T; Rázga Zs; Tiszlavicz L; Virág L; Jost N; Tóth A; Papp JGy; Leprán I; Varró A; Nagy N.
CARDIOLOGIA HUNGARICA 48:Suppl.C p. C48 (2018)

II. Effects of ORM-10962, a novel inhibitor of sodium/calcium exchanger, on ion currents of canine isolated cardiomyocytes, and its antiarrhythmic efficacy in guinea pigs

Horváth A, Kohajda Zs, **Morvay N**, Levijoki J, Leprán I, Papp JGy, Varró A, Virág L, Jost N.
CARDIOLOGIA HUNGARICA 44:Suppl.E p. E27 (2014)

III. ORM10962, a selective sodium-calcium exchange inhibitor, protects against ouabain induced arrhythmias in anesthetized guinea-pigs.

Morvay N, Jost N, Varró A, Papp JGy, Possesello P, Levijoki J, Otsomaa L, Koskelainen T, Leprán I.
BASIC & CLINICAL PHARMACOLOGY & TOXICOLOGY 115:Suppl.1 pp. 39-40.,2 p. (2014)

IV. Effects of ORM-10962, a novel inhibitor of sodium/calcium exchanger, on ion currents of canine cardiomyocytes, and its antiarrhythmic action in guinea pigs.

Jost N, Kohajda Zs, Horváth A, **Morvay N**, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Leprán I, Papp JGy, Virág L, Varró A.
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I. Evaluation of the potential antiarrhythmic effect of fish meat during myocardial ischaemia – reperfusion in rats

Morvay N, Szűcsné Peter J, Kostadinović LM, Đuragić OM, Kokić BM, Csengeri I, Lepran I

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III. Investigation of cardiovascular effeccts of long-term endurance exercise training in rabbits and dogs (Hosszú időtartamú állóképességű tréning kardiovaszkuláris hatásainak vizsgálata nyúlban és kutyában)

Polyák A, Kui P, **Morvay N**, Leprán I, Ágoston G, Varga A, Baczkó I, Farkas A, Papp JGy, Varró A, Farkas A

CARDIOLOGIA HUNGARICA 47: Suppl.G pp. G40-46. , 7 p. (2017)

IV. Long-term endurance training-induced cardiac adaptation in new rabbit and dog animal models of the human athlete's heart

Polyák A, Kui P, **Morvay N**, Leprán I, Ágoston G, Varga A, Nagy N, Baczkó I, Farkas A, Papp JGy, Varró A, Farkas AS

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2. ACRONYMS AND ABBREVIATIONS

APD – Action potential duration

AF – Atrial fibrillation

AMIO – Amiodarone

AP – Action potential

AVbl – Atrio-ventricular block

Ca^{2+} – Calcium ion

Cl^{-} – Chloride ion

CVD – Cardiovascular diseases

DAD – Delayed afterdepolarization

DEA – Desethylamiodarone

EAD – Early afterdepolarization

I_{CaL} – Voltage-gated L-type calcium current

I_{Kr} – Rapid component of the delayed rectifying potassium current

I_{Ks} – Slow component of the delayed rectifying potassium current

I_{K1} – Inward rectifying potassium current

I_{NCX} – Sodium-calcium exchanger current

i.p. – Intraperitoneally

I/R – Ischaemia/reperfusion

K^{+} – Potassium ion

MI – Myocardial infarction

Na^{+} – Sodium ion

NCX – Sodium-calcium exchanger

NHE – Sodium-hydrogen exchanger

ROS – Reactive oxygen species

TdP – Torsades de Pointes

TDR – Transmural dispersion of repolarization

VEB – Ventricular extrasystole and bigeminia

VF – Ventricular fibrillation

VT – Ventricular tachycardia

VPB – ventricular premature beat

3. SUMMARY

Cardiovascular diseases (CVD) are the leading cause of death, resulting in 30% of all death worldwide. This incidence is even higher in Europe, which can promote the development of new antiarrhythmic drugs. Amiodarone (AMIO) is a widely used antiarrhythmic drug in the clinical practice but have many extracardiac adverse effects. Its active metabolite, desethylamiodarone (DEA) produces very similar pharmacological effects, thereby seems to be a potential antiarrhythmic drug. The Na^+/Ca^{2+} exchanger (NCX) plays important role in the Ca^{2+} homeostasis of the heart, as well as in the development of Ca^{2+} overload-related arrhythmias. Therefore, specific inhibition of NCX is thought to be effective therapeutic tool against arrhythmias (early- and delayed afterdepolarization (EAD, DAD)). The aim of the present investigations was to elucidate the potential antiarrhythmic effects of these mechanisms in male Sprague-Dawley rats and Hartley guinea-pigs in different *in vivo* models.

DEA, similarly to its parent compound AMIO, significantly improved the survival rate as well as delayed the onset of the first arrhythmias during the acute phase of experimental myocardial infarction in a conscious rat model. Chronic oral DEA treatment (using half of the dose of AMIO) resulted in similar myocardial tissue DEA concentration as with the double dose of AMIO. It is expected that substituting DEA for AMIO in clinical practice would result in a better therapeutic option, producing similar effectiveness with less toxicity.

Pretreatments with selective NCX blockers proved to be effective against DAD related arrhythmogenesis in an *in vivo* ouabain-induced arrhythmia model in guinea-pigs, as demonstrated by the delay of the development of ventricular arrhythmias. On the other hand, NCX inhibition failed to exert cardioprotection against ischaemia/reperfusion-induced arrhythmias in rats.

According to our results, both DEA and the selective NCX inhibitor ORM 10962 seem to be possible therapeutic tools against arrhythmias in the clinical practise.

4. INTRODUCTION

4.1 Epidemiology of cardiovascular diseases

Cardiovascular diseases (CVD) are the leading cause of death both in developed and in developing countries. According to the Global Burden of Disease Study, 30% of all death worldwide were caused by CVD (Naghavi et al., 2015). This incidence is even higher in Europe and CVD are responsible for over 4 million deaths per year in Europe, close to half of all death (Townsend et al., 2015). A common precipitating factor of CVD are arrhythmias. This high prevalence and mortality rate promoted the development of a large number of antiarrhythmic drugs in order to prevent life-threatening arrhythmias and associated subsequent deaths.

However, it revealed that most antiarrhythmic drugs have arrhythmogenic properties, i.e. worsening or directly causing arrhythmias due to the influence of the cardiac depolarization and repolarization in the asymptomatic state of the diseased heart (proarrhythmic effect). Drugs that slow the conduction can promote the development of reentry. One of the largest arrhythmia study (CAST: Cardiac Arrhythmia Suppression Trial) demonstrated the mortality increasing effect of the class I/C I_{Na} blocker flecainide in patients after myocardial infarction (MI). In the background of the increased mortality the abnormal slowing of impulse conduction in the heart was assumed (Cardiac Arrhythmia Suppression Trial, 1989). Another study, the SWORD trial (Survival With Oral D-sotalol) verified an increased mortality of the patients after MI receiving d-sotalol, a pure class III antiarrhythmic drug (Waldo et al., 1996). It is concluded that drugs prolonging cardiac action potential duration (ADP) (e.g. class III antiarrhythmic drugs) can sometimes induce ventricular premature beats (VPB) due to the development of early afterdepolarization (EAD), causing polymorphic ventricular tachycardias, i.e. Torsades de Pointes (TdP). Other drugs were also proved to cause arrhythmias: drugs increasing the intracellular Ca^{2+} level, e.g. digitalis, can provoke delayed afterdepolarization (DAD), leading to ventricular tachycardia.

4.2 Arrhythmias

Arrhythmias are deviations from the normal sinus rhythm of the heart. Pathological changes of the cellular electrical activity were found in the background of the development of arrhythmias. The electrical activity of cardiac tissue is determined by the highly regulated flow of ions across the cell membrane during the cardiac action potential, mediated by several ion channels, currents as well as transport proteins (Roden & George, 1996). The relatively long cardiac action potential (AP), resulting in a relatively long refractory period is very important in the prevention of cardiac arrhythmias. The refractory period depends on the length of the repolarization and the capability for reactivation of the Na^+ and Ca^{2+} channels. If the depolarizating currents re-activate earlier – VPBs can occur on the base of triggered activity.

Triggered activity is a special form of the abnormal automaticity, the premature activation of cardiac tissues by afterdepolarization, oscillation in membrane potential following the preceding action potential (trigger) (Tse, 2016; Waldo & Wit, 1993). Afterdepolarizations are divided into two types according their occurrence during the AP: EAD and DAD (Figure 1). Both of them are the results of the elevated intracellular Ca^{2+} concentration and play a significant role in arrhythmogenesis.

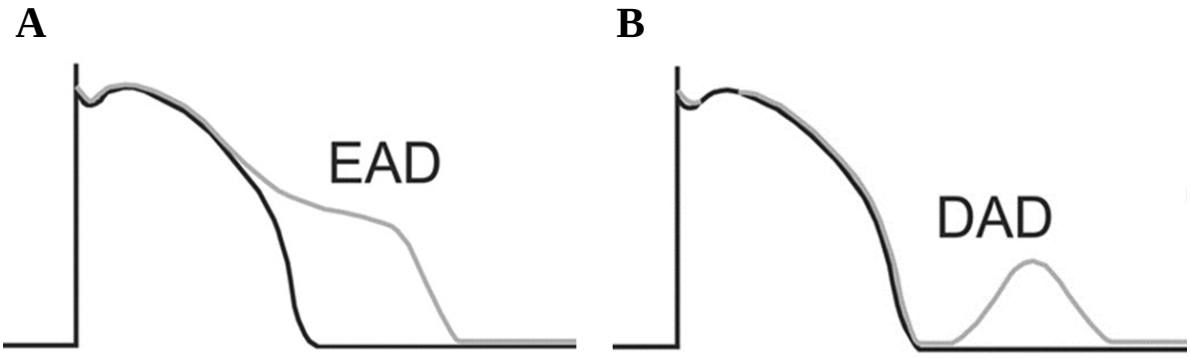


Figure 1. Early- and delayed afterdepolarizations (EAD and DAD). **A**: L-type Ca^{2+} currents reactivates due to prolonged action potential duration, leading to the production of VPBs
B: Increased intracellular level of Ca^{2+} increases the driving force of the electrogenic reverse Na^+/Ca^{2+} exchanger leading to DADs. If DADs reach the threshold potential, a triggered beat can arise, causing a VPB. Modified from Khan, 2004.

Another important arrhythmia component is the reentry. Reentry is related to the dysfunction of the conduction, based on the re-entered stimuli which is failed to terminate itself after the repolarization of a normal AP (circus movement), resulting in VPB or tachycardia.

It is well known, that arrhythmias require both initiating (trigger) and maintaining mechanisms. The initiating mechanism can be either an EAD or DAD leading to VPBs that might trigger re-entrant tachyarrhythmias. The maintaining mechanism has been described as the '4 dimensions' of dispersion of cardiac repolarization in space and time: transmural dispersion of repolarization (TDR); apico-basal dispersion; interventricular dispersion and temporal dispersion. Temporal component of dispersion includes the instability of subsequent APs. Dispersion leads to increased heterogeneity of refractoriness, which sets the stage for reentry arrhythmia generation and maintenance (Farkas & Nattel, 2010).

Arrhythmias may be described by rate (bradycardia/tachycardia), regularity (regular/irregular), its origin (atrial/ventricular) or duration (sustained/non-sustained).

4.3 Ischaemia/reperfusion induced arrhythmias

Ischaemia means a reduced blood supply to the heart, that is caused by the narrowing or occlusion of the coronary artery. If it is maintained for several minutes, severe arrhythmias and contractile failure is developing. Longer ischaemia, lasting for hours is resulting in myocardial infarction, characterized by irreversible tissue damage. The restoration of the blood flow, i.e. reperfusion, is a generally accepted method in case of myocardial ischaemia, to reduce the irreversible damage of cells. However, reperfusion itself can be harmful due to the Ca^{2+} overload, reactive oxygen species (ROS) formation and accumulation, the rapid change of the intracellular pH and the mitochondrial permeability. All of these changes together can cause endothelial dysfunction, reversible loss of myocardial contractility (myocardial stunning), arrhythmias, necrosis and apoptosis, which are known as reperfusion injury (Frank et al., 2012; Yellon & Hausenloy, 2007). The above-mentioned electrophysiological changes can induce different type of arrhythmias; reentry due to the slowed conduction, unidirectional block or inhomogeneity in the refractory period and/or triggered activity due to Ca^{2+} overload.

4.4 Antiarrhythmic therapies

During the last decades, the still high rate of cardiac arrhythmia-related death has been promoted the innovations of both interventional (catheter ablation, implantable cardioverter defibrillator (ICD)) and pharmacological therapies.

The targets of pharmacological intervention are 1) the suppression of the automaticity (decreasing the ectopic activity) by the inhibition of the Na^+ and Ca^{2+} channels, 2) the lengthening of the refractory period (terminating the reentry), 3) the reduction of triggered activity and 4) the decrease of the activity of the sympathetic autonomous nervous system.

4.4.1 Amiodarone-Desethylamiodarone

According to the Vaughan Williams classification, amiodarone (AMIO, iodine-rich benzofuran derivative) is a representative of the class III antiarrhythmic drugs, with complex antiarrhythmic effects. The main effects of AMIO are the prolongation of the APD and the inhibition of the K^+ channels, whereas it can inhibit other ion channels, e.g. cardiac voltage dependent sodium-, and calcium-channels, as well as produce a β-adrenergic blocking effect (Kodama et al., 1999) on cardiomyocytes. The short- and long-term administration of amiodarone have different cardiac effects. Short-term effects include the inhibition of the Na^+ and Ca^{2+} channels, while no consistent effect on the APD. Long-term administration inhibits the inward calcium current less, but increases the APD (through a decrease in I_K and I_{to} density) and the refractory period more than after a short-term treatment (Drvota et a., 1998). However, AMIO exerts serious extracardiac adverse effects, e.g. pulmonary fibrosis, hepatotoxicity, photodermatosis, cornea deposits, probably due to its unique pharmacokinetic properties (extremely long elimination half-life and its metabolic interactions with several other drugs) (Tisdale et al., 1995).

During AMIO treatment an electrophysiologically active metabolite, desethylamiodarone (DEA) is produced (Figure 2) which accumulates in the blood plasma and tissues, including the myocardium (Flanagan et al., 1982). DEA produces very similar pharmacologic effects to AMIO. During the administration of AMIO, the electrophysiologic effects of both AMIO and DEA are likely to be additive (Kato et al., 1988).

Acute administration of both the AMIO and DEA significantly prolonged the QRS, QT and QTc intervals of the electrocardiogram in rats. DEA showed a significantly greater potency in this respect (Nattel, 1986). Acute treatments with either AMIO or DEA suppressed ventricular arrhythmias after a two-stage coronary artery ligation in dogs (Nattel et al., 1988). Long-term administration of AMIO or DEA in rabbits prolonged the APD and the effective refractory period (Kato et al., 1988). However, there are no data available comparing the effects of a long-term treatment with AMIO and DEA on arrhythmias under *in vivo* conditions.

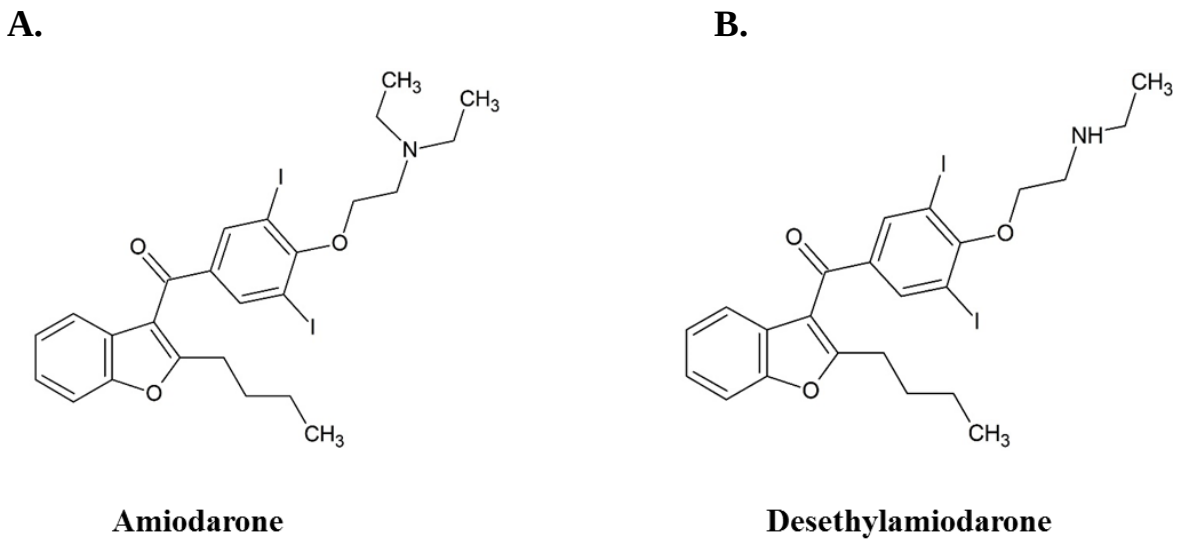


Figure 2. Chemical structure of amiodarone (**A**) and desethylamiodarone (**B**)

4.5 Sodium/calcium exchanger

The Na^+/Ca^{2+} exchanger (NCX) is considered the main transmembrane intracellular Ca^{2+} remover in the cardiac cell. During systole, the elevated cytosolic Ca^{2+} concentration induces NCX to extrude 1 Ca^{2+} from the cytosol into the extracellular space, transporting 3 Na^+ from the extracellular space to the cytosol thereby resulting in a net positive charge accumulation in the intracellular space. This ionic exchange generates an inward current (I_{NCX}) leading to an increase in net I_{Na} which delays repolarization. Therefore, increased I_{NCX} – by prolonging repolarization – can lead to the formation of DAD, triggering tachyarrhythmias (Antoons et a., 2012; Pogwizd & Bers, 2004). On the other hand, reduced I_{NCX} would decrease $I_{Na,}$ thus APD shortens, leading to decreased formation of triggered arrhythmias (Pott et al., 2011).

NCX plays an important role in ischaemia/reperfusion (I/R) injuries, as well. Under pathological conditions, the intracellular Na^+ level raises due to the inhibition of the Na^+/K^+ -ATPase. During high intracellular Na^+ concentration the NCX is working in a reverse mode, resulting in the elevation of intracellular Ca^{2+} concentration (Imahashi et al., 1999; Van Emous et al., 1998; Xiao & Allen, 1999). Due to the anaerobic metabolism under ischaemic conditions the pH of the intracellular compartment shifts to acidosis. These changes lead to the activation of the Na^+/H^+ exchanger (NHE), increasing further the net intracellular Na^+ level. This process further activates the reverse mode of NCX, resulting in a Ca^{2+} overload, which can cause cell injury and myocardial stunning (Imahashi et al., 2005; Kusuoka, 1999; Lee & Hryshko, 2004).

In the clinical practice, class I/B antiarrhythmic drugs (lidocaine, phenytoin), or intravenously administered magnesium can be applied for the elimination of DAD-induced arrhythmias. The specific inhibition of NCX is thought to be an effective therapeutic tool against Ca^{2+} overload related arrhythmias (EAD, DAD) (Nagy et al., 2004). Drugs inhibiting the influx of both Na^+ and Ca^{2+} may prevent the occurrence of DAD-induced arrhythmias.

KB-R7943 and SEA 0400 are two widely investigated NCX blockers (Amran et al., 2004). SEA 0400 (Figure 3) seems to be 100 times more selective than KB-R7943, without the inhibition of Na^+ and K^+ ion channels, while inhibiting Ca^{2+} channels (Birinyi et al., 2005; Matsuda et al., 2001). Although neither of them is completely selective, their antiarrhythmic effects have been elucidated in different *in vitro* as well as *in vivo* animal models. Both KB-R7943 and SEA 0400 exert protective effects due to the limitation of the infarct size after coronary artery occlusion/reperfusion (Magee et al, 2003), as well as decreasing the incidence of ventricular arrhythmias (Nakamura et al., 1998; Takahashi et al., 2003; Nagasawa et al., 2005).

SEA 0400 also effectively decreases the occurrence of early and delayed afterdepolarizations in canine isolated cardiac tissues (Nagy et al., 2004). However, the antiarrhythmic effect is controversial and negative results are also found (Takahashi et al., 2004) e.g. SEA 0400 did not decrease the incidence of TdPs in an *in vitro* TdP rabbit model (Farkas et al., 2009).

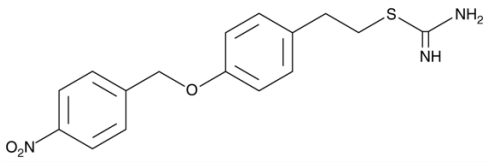
In an aconitine-induced arrhythmia model KB-R7943 suppressed the triggered activity, whereas SEA 0400, in the highest dose (10mg/kg) was ineffective (Amran et al., 2004). KB-R7943 did not only suppress the NCX, but also the Na^+ and Ca^{2+} channels, preventing from Na^+ and Ca^{2+} overload. On the other hand, SEA 0400, because of its better potency and selectivity, did not influence the Na^+ and Ca^{2+} channels, thus did not suppress the development of aconitine triggered arrhythmias. These results may suggest that the prevention of Na^+ overload may be more important for the cardioprotection in this model, than the prevention of Ca^{2+} overload.

Although, these NCX blockers are thought to be selective, KB-R7943 and SEA 0400 influence other transmembrane ionic currents as well, including the I_{Na}, I_K and the I_{CaL}, respectively. The inhibition of these ionic currents alone may exert also an antiarrhythmic effect; therefore, it is still questionable to what extent the NCX inhibition is responsible for the cardiac protection.

Recently, a more potent and more selective NCX blocker, ORM 10103 (Figure 3), has been developed which could supress the pharmacologically-induced EADs and DADs without influencing several other ion channels, e.g. Ca^{2+}, Na^{+} and K^{+} channels (Jost et al., 2013). ORM 10103 is effective *in vitro* against arrhythmias, caused by higher intracellular Na^{+} level-induced intracellular Ca^{2+} elevation, without influencing the AP waveform (Nagy et al., 2014).

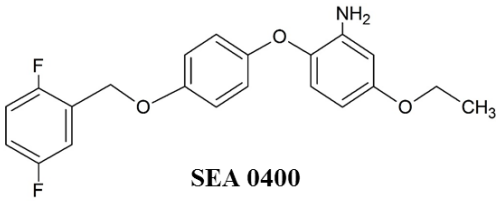
More recently a derivative of ORM 10103, also highly selective NCX inhibitor (ORM 10962) (Figure 3) became in focus of researches. Some in vitro experimental results (Kohajda et al., 2016) prove the effectiveness and selectivity of ORM 10962.

A.



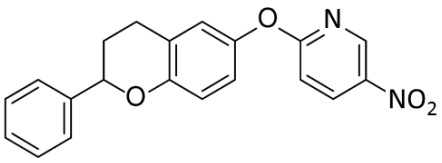
KB-R7943

B.



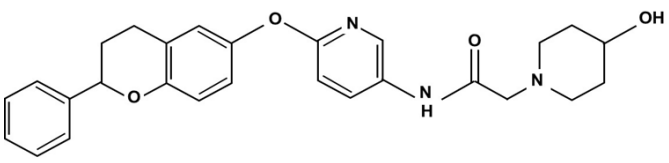
SEA 0400

C.



ORM 10103

D.



ORM 10962

Figure 3. Chemical structure of different NCX inhibitors

4.6 Cardiac glycosides

Cardiac glycosides (e.g. digoxin, ouabain) have a positive inotropic effect so they are widely used to improve the force of the cardiac contraction in failing heart. However, they often induce arrhythmias due to the excess inhibition of the Na^+/K^+-ATPase, leading to the excess accumulation of intracellular Ca^{2+}. It has been found that NCX blockers such as KB-R7943 and SEA 0400 have protective effects against ouabain-induced arrhythmias in guinea-pigs (Amran et al., 2012; Watano et al., 1999). Due to the questionable selectivity of these inhibitors, it is very speculative to assume that this effect is mediated by the selective inhibition of NCX. A highly selective NCX inhibitor (i.e. ORM 10962) could help to elucidate this question.

4.7 Aims of the study

The aims of the present investigations were to elucidate:

1. the effectiveness of the long-term pretreatment with AMIO or with its active metabolite, DEA, on ventricular arrhythmias, induced by acute myocardial infarction in conscious rats.

2. the effects of highly selective and effective NCX inhibitor ORM 10962, and to compare with the effects of SEA 0400 on ouabain- or ischaemia/reperfusion-induced cardiac arrhythmias *in vivo*.

5. MATERIALS AND METHODS

Investigations were performed according to the Ethical Committee for Protection of Animals in Research of the University of Szeged, Hungary (permit No. I-74-9/2009), conform with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No. 85-23, revised 1996) and were approved by the Csongrád County Governmental Office for Food Safety and Animal Health, Hungary (approval No.: XIII/1211/2012).

5.1 Animals

Male Sprague-Dawley CFY rats (weighing 260-330 g) and Hartley guinea-pigs (weighing 320-440 g) were used. The animals were fed with standard chow for rats or guinea-pig, respectively (Szindbád Kft., Gödöllő, Hungary), and were allowed to drink tap water *ad libitum.* 12 h dark/light cycles were maintained.

5.2 Coronary artery ligation-induced arrhythmias in conscious rats

Coronary artery occlusion was performed in conscious rats as described earlier (Leprán et al., 1983). In a preliminary surgery under ether anaesthesia, we opened the chest in the 4th intercostal space. A loose silk loop (5-0, Ethicon, Great Britain) was led around the left main coronary artery, and then we closed the chest. Small needle-electrodes were implanted subcutaneously in order to register the ECG later. After the complete recovery and healing of the animals (7-8 days after the preliminary surgery), the loose silk loop was tightened to occlude the coronary artery in conscious, freely moving animals. During the first 15 min of myocardial infarction (MI) a bipolar ECG was recorded continuously (PowerLab 8SP, ADInstruments, Great Britain).

The survival rate during the acute phase (first 15 min) and during the following 16 hours after coronary artery occlusion was followed (Figure 4). The incidence and duration of arrhythmias in the acute phase were evaluated according to the Lambeth Conventions, i.e. ventricular fibrillation (VF), ventricular tachycardia (VT), and other types of arrhythmias, including ventricular extrasystoles and bigeminias (VEB) (Walker et al., 1988).

An arrhythmia score was given, including the type and duration of different arrhythmias by giving a grade to each animals (Table 1) (Leprán & Szekeres, 1992).

Table 1. Arrhythmia score given according to the type and duration of arrhythmias

Score	Incidence and duration of arrhythmia
0	no arrhythmia
1	< 10 sec VEB and/or VT
2	11-30 sec VEB and/or VT
3	31-90 sec VEB and/or VT
4	91-180 sec VEB and/or VT
5	>180 sec VEB and/or VT; >10 sec VF
6	irreversible VF

The size of the myocardial infarction was quantified (Image J software, NIH, Bethesda, USA) in the animals surviving for 16 hours after coronary artery occlusion using 0.1% nitrotetrazolium-blue dye staining (solved in Sörensen phosphate buffer, pH=7.4), as to demonstrate appropriate production of MI. Hearts were excised, washed with NaCl (0.9%) and sliced. The slices were placed into the staining solution, and after 10 minutes were scanned. Animals with a scar tissue of < 5% were excluded from further evaluation.

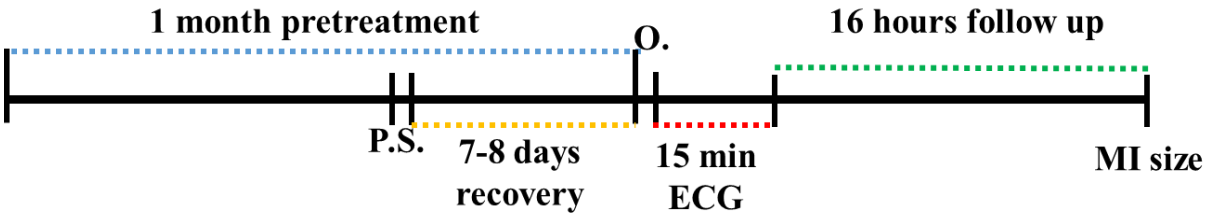


Figure 4. Protocol of coronary artery ligation-induced arrhythmias in conscious rats
P.S.: preliminary surgery; O.: occlusion of the left main coronary artery

5.3 Myocardial ischaemia/reperfusion-induced arrhythmias in anesthetized rats

Male Sprague-Dawley rats were used for the experiments. Coronary artery occlusion and reperfusion were performed as described previously (Baczkó et al., 1997; Leprán & Szekeres, 1992). During pentobarbitone anaesthesia (60 mg/kg intraperitoneally) the chest was opened between the fourth and fifth ribs and the heart was exposed. A loose loop of atraumatic silk (4/0 MERSILK black, W582, ETHICON, Edinburgh, Great Britain) was placed around the left main coronary artery. After the surgery artificial respiration was started (at a rate of 60 strokes/min, volume 0.7 ml/stroke/100 g; Harvard Rodent Ventilator 683, South Natick, MA, USA). After 10 minutes normalization we occluded the coronary artery by tightening the loop for 6 minutes, followed by a reperfusion period for 5 minutes (Figure 5). Arterial blood pressure was measured via the right arteria carotis communis. The left vena femoralis was prepared and cannulated for drug administration.

In both protocols the ECG was recorded (Figure 6). The survival rate and incidence of arrhythmias was determinated. No attempt was made artificially to revert VF during ischemia or reperfusion.

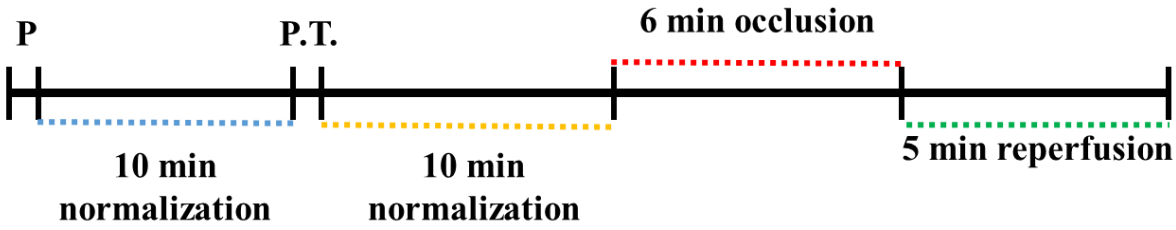


Figure 5. The protocol of the myocardial ischaemia-reperfusion induced arrhythmias in rats. P: preparation, P.T.: pretreatment (SEA 0400/ORM 10962)

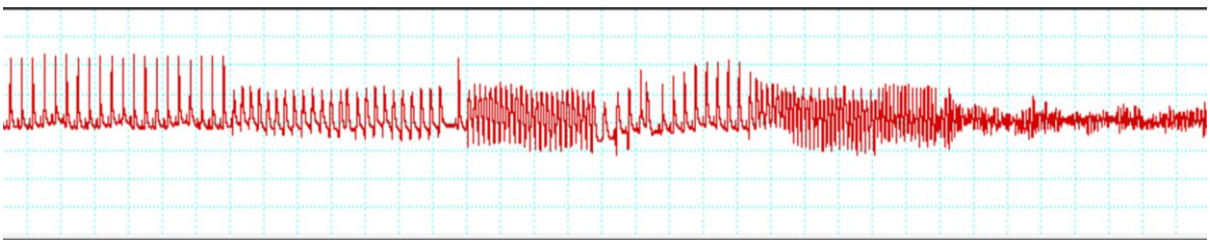


Figure 6. Representative ECG of ventricular arrhythmias during reperfusion after 6 min coronary artery occlusion in rat

5.4 Ouabain-induced arrhythmias in guinea-pigs

5.4.1 Prevention of ouabain-induced arrhythmias

We used a method described by Thomas & Tripathi (1986) and Watano et al. (1999). Male guinea-pigs, weighing 320-440 g, were anaesthetized with pentobarbitone (45 mg/kg intraperitoneally). The trachea was prepared, cannulated and the animals were ventilated with a respirator with room air (volume 1.5 ml/100g at a rate of 35 strokes/min Harvard Rodent Ventilator 683, South Natick, MA, USA). The right arteria carotis communis was prepared and cannulated in order to measure the arterial blood pressure (PowerLab 8 SP, ADInstruments, Great Britain). The left vena jugularis was cannulated for the drug administration and later for the ouabain infusion. Ouabain was continuously infused at the rate of 10 µg/kg/min using an infusion pump, at the rate of 6ml/h/kg, (Terumo, TE-331 Syringe pump, Leuven, Belgium) intravenously via a polyethylene cannula (Figure 7).

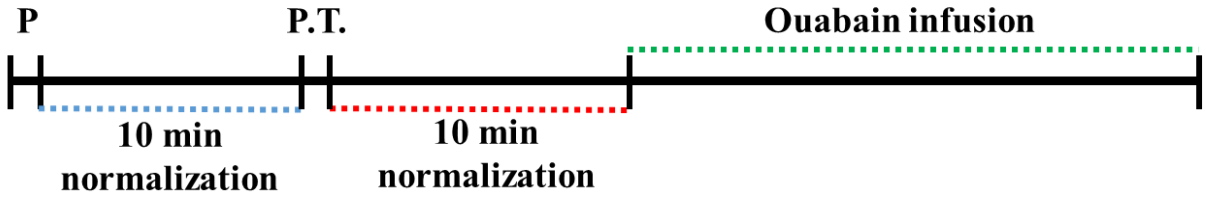


Figure 7. Protocol of the ouabain-induced arrhythmia in guinea-pigs.
P: preparation, P.T.: pretreatment (SEA 0400/ORM 10962)

5.4.2 Suspension of ouabain-induced arrhythmias

The above-described preparation was performed with the modification that ORM 10962 was administered intravenously after the termination of ouabain infusion (10 µg/kg/min) at the 16th min (Figure 8).

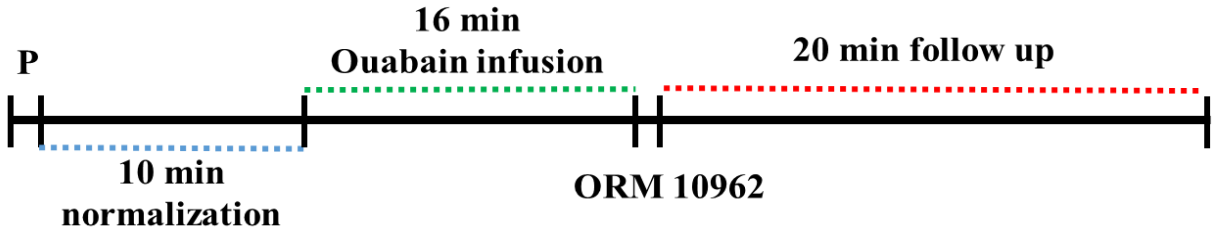


Figure 8. Protocol of the suspension of ouabain-induced arrhythmia in guinea-pigs.
P: preparation

5.5 Determination of the plasma and tissue concentration of amiodarone and desethylamiodarone

At the end of the feeding period the rats were anesthetized with pentobarbitone (60 mg/kg intraperitoneally) and blood was drawn from the abdominal aorta and the heart was excised. The ventricular myocardium was homogenized in 0.01M KH_2PO_4 (pH=4.3): methanol = 75:25 mixture (1:4 w/v) with a tissue blender. Plasma and tissue homogenates were stored at -70 °C until later analysis. AMIO and DEA was determined according to Bolderman et al. (2009), using a HPLC apparatus (Shimadzu Corporation, Kyoto, Japan) equipped with Kromasil Eternity C18 (5µm, 150 mm x 4,6 mm AkzoNobel, Bohus, Sweden) analytical column.

5.6 Drugs and pretreatments

Amiodarone was purchased from Sequoia Research Products Ltd. (Pangourne, UK), desethylamiodarone from Szintekon Ltd. (Debrecen, Hungary), ouabain from Sigma-Aldrich Fine Chemicals, (St. Louis, MO, U.S.A.), verapamil from Zentiva (Sanofi Group, Prague, Czech Republic). SEA 0400 was synthesized by Department of Pharmaceutical Chemistry, University of Szeged. ORM 10962 was a gift from Orion Pharma, (Espoo, Finland).

5.6.1 Amiodarone and desethylamiodarone pretreatments

In rats, one-month oral pretreatment was applied before the coronary artery occlusion. The applied doses were as follows: AMIO 30 or 100 mg/kg/day (loading doses of 100 or 300 mg/kg for 3 days); DEA 15 or 50 mg/kg/day (loading doses of 100 or 300 mg/kg for 3 days). Control animals received the vehicle (0.5 % metylcellulose : tap water 1:4) in a volume of 5ml/kg.

5.6.2 SEA 0400 and ORM 10962 pretreatments

SEA 0400 or ORM 10962 was administered in a bolus intravenous injection 10 minutes before starting ouabain infusion in guinea pigs or 10 minutes before coronary artery occlusion in rats. SEA 0400 (1 mg/kg) and ORM 10962 (0.3 mg/kg) were solved in DMSO : HCl (0.1M) : 0.9% NaCl, 1:1:8, and DMSO : 0.9% NaCl, 1:9, respectively. The control group received the vehicle of the drugs and we used verapamil (Ca^{2+} channel blocker) as a positive control (0.32 mg/kg intravenously).

5.7 Statistical analysis

The survival rate was compared by using the χ^2-method with Yates-correction. All other parameters were expressed as mean±standard error of the mean (SE) and after analysis of variance were compared by means of the modified ‘t’-statistical method (Wallenstein et al., 1980).

6. RESULTS

6.1 Comparing the antiarrhythmic effects of amiodarone and desethylamiodarone in vivo

6.1.1 Coronary artery ligation-induced arrhythmias in conscious rats

Neither AMIO nor DEA administration produced any visible behavioral changes of the animals during the long-term treatment, or any difference in the gaining of body weight.

Coronary artery occlusion in conscious rats within 4-6 minutes resulted in various arrhythmias (ventricular extrasystole, bigeminia, ventricular tachycardia), leading frequently to irreversible ventricular fibrillation (Figure 9). The larger doses of both pretreatments (AMIO and DEA) significantly improved the survival rate during the acute phase of experimental myocardial infarction (Table 2). Interestingly, some animals did not develop arrhythmias at all during the first 15 min after coronary artery occlusion, in spite of the characteristic ECG changes (e.g. the QRS-complex distortion and T-wave elevation) and developing myocardial damage 16 hours later (Figure 9).

Table 2. Survival rate and incidence of ventricular arrhythmias during 15 min coronary artery occlusion in conscious rats

Groups	N	Survived		Incidence of arrhythmias (n/%)					Arrhythmia score
		n	%	None	VF	VT	VEB	Br	
Control	39	12	31	2/5	29/74	35/90	28/72	1/3	4.77±0.33
AMIO 30	23	9	39	1/4	16/70	20/87	13/57	6/26 *	4.57±0.43
DEA 15	23	8	35	3/13	16/70	18/78	14/61	3/13	4.35±0.52
AMIO 100	22	18 **	82	10/46 ***	5/23 ***	9/41 ***	8/36 **	2/9	2.05±0.52 ***
DEA 50	22	14 *	64	6/24 *	10/46 *	15/68 *	13/59	2/9	3.27±0.56 *

N: total number of animals; n: number of the animals with the given response; %: percentage of the animals with the given response
None: animals without any arrhythmias; VF: ventricular fibrillation; VT: ventricular tachycardia; VEB: ventricular extrasystole and bigeminia. Asterisks show statistically significant difference compared to the vehicle-treated controls *P<0.05; **P<0.01; ***P<0.001

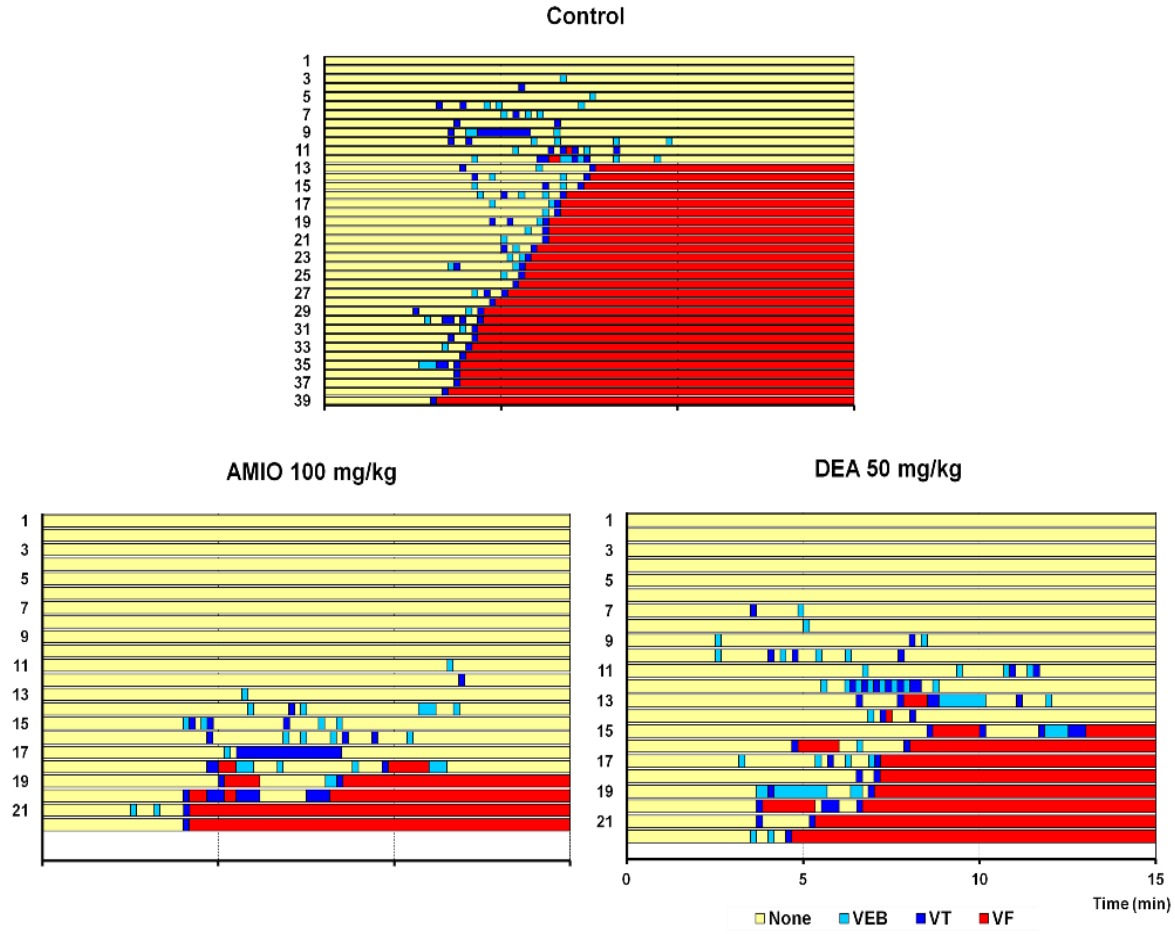


Figure 9. Arrhythmia map of coronary artery occlusion/reperfusion-induced arrhythmias in conscious rats of control, amiodarone and desethylamiodarone pretreated groups.
None: no arrhythmia; VEB: ventricular extrasystole and bigeminia; VT: ventricular tachycardia; VF: ventricular fibrillation. Each row represents a single animal and its "arrhythmia history".

The larger dose of both AMIO and DEA pretreatments significantly delayed the onset time of the first arrhythmias (Table 3). However, the mean duration of the arrhythmic attacks in the surviving animals did not change significantly.

Table 3. Influence of long-term amiodarone (AMIO) or desethylamiodarone (DEA) pretreatment on the onset time and duration of arrhythmias after coronary artery occlusion in conscious rats surviving the acute phase of experimental myocardial infarction.

Group		Arrhythmia		Duration of arrhythmic attacks (sec)				
		first (min)	last (min)	VF	VT	Other	Br	Total
Vehicle	Mean	4.72	9.00	3	17	18	3	41
	SE	0.44	0.98	1.8	4.7	5.1	3.3	9.8
	n	12	12	12	12	12	12	12
AMIO 30 mg/kg	Mean	5.17	9.37	6	9	18	10	42
	SE	0.52	0.87	3.8	2.6	4.3	6.5	6.0
	n	9	9	9	9	9	8	9
AMIO 100 mg/kg	Mean	9.95	12.75	2	16	12	2	32
	SE	1.10	0.70	1.7	10.1	5.0	1.7	12.4
	n	18	18	18	18	18	18	18
		*	*					
DEA 15 mg/kg	Mean	5.86	11.06	10	4	15	11	40
	SE	0.80	1.29	10.0	1.8	7.1	11.3	17.7
	n	8	8	8	8	8	8	8
DEA 50 mg/kg	Mean	7.56	11.30	5	16	19	6	46
	SE	1.05	1.01	3.4	6.4	9.3	5.7	15.8
	n	14	14	14	14	14	14	14
		*						

Values are mean±S.E. For other details see Table 2.

6.1.2 Heart rate changes in conscious rat MI model

The mean values of the baseline heart rate did not differ among the control and pretreated groups before the coronary artery occlusion. A modest elevation in the heart rate was visible as a control response, during the coronary artery occlusion. This moderate tachycardia developed in each treated groups and did not differ significantly from the control (Table 4).

Table 4. Influence of long-term amiodarone (AMIO) or desethylamiodarone (DEA) pretreatment on the heart rate (beats/min) in conscious rats.

Group		Baseline	Time after coronary artery occlusion (min)						
			1	3	5	7	10	12	15
Vehicle	Mean	327	402	395	394	390	380	382	370
	SE	5.3	9.4	7.5	8.9	11.7	15.5	15.1	11.2
	n	39	37	36	26	12	12	12	12
AMIO 30 mg/kg	Mean	343	415	401	385	393	360	364	382
	SE	8.1	12.7	17.4	14.7	17.7	21.3	18.9	23.4
	n	23	23	19	15	9	9	9	9
AMIO 100 mg/kg	Mean	321	373	363	356	365	357	348	340
	SE	5.1	12.2	12.9	13.8	9.9	12.1	9.3	7.9
	n	22	22	22	17	17	17	18	18
DEA 15 mg/kg	Mean	343	420	400	397	410	393	386	375
	SE	7.9	15.7	14.9	14.8	16.1	11.9	13.2	12.0
	n	23	21	20	15	8	8	8	8
DEA 50 mg/kg	Mean	330	398	382	369	365	363	359	350
	SE	8.9	11.4	10.3	9.9	8.5	10.8	9.4	6.2
	n	22	21	22	18	15	15	14	14

Baseline: 5 min before coronary artery occlusion. Values are mean ± S.E. n: number of animals at the given time point.

6.1.3 Amiodarone and desethylamiodarone concentrations in the plasma and myocardium

At the end of the pretreatment period, we determined the plasma and the myocardium concentration of AMIO and DEA. As a result of AMIO treatment (100 mg/kg/day) the plasma concentration of its active metabolite (i.e. DEA) was about 1/4 of the parent molecule (AMIO). The tissue concentration of AMIO in the myocardium was about 10-times higher ($P<0.05$) than in the plasma which was equivalent to the tissue concentration of DEA (Table 5). Long-term oral DEA (50 mg/kg/day) treatment resulted in similar myocardial tissue DEA concentration to that measured after AMIO (100 mg/kg) pretreatment (Table 5).

Table 5. The plasma and myocardial concentration of amiodarone (AMIO) and its metabolite, desethylamiodarone (DEA) after long-term oral administration of AMIO or DEA in rats.

Group		Plasma (μg/ml)		Myocardium (μg/g)	
		AMIO	DEA	AMIO	DEA
Vehicle	Mean	0.00	0.00	0.00	0.00
	SE	0.00	0.00	0.00	0.00
	n	4	4	4	4
AMIO	Mean	0.68	0.15	7.91	8.95
100 mg/kg	SE	0.10	0.03	1.25	2.21
	n	12	11	30	30
DEA	Mean	0.00	0.20	0.00	7.35
50 mg/kg	SE	0.00	0.02	0.00	0.73
	n	16	16	27	27

Values are expressed as mean ± S.E.; n: number of the samples

6.2 Investigation of the antiarrhythmic effects of sodium/calcium exchange inhibition

6.2.1 Effects of SEA 0400 and ORM 10962 pretreatments on coronary artery occlusion/reperfusion-induced arrhythmias in rats

Coronary artery occlusion in anesthetized rats resulted in sporadic arrhythmias starting around the 5th minutes of ischaemia. The 6-minutes-long occlusion rarely produced life-threatening arrhythmias, however, reperfusion after this duration of ischaemia rapidly induced severe arrhythmias leading to death in some of the rats. One-third of the control animals (7 out of the 21) died due to irreversible ventricular fibrillation. Other serious arrhythmias, e.g. tachycardia, frequently occurred. Interestingly, ventricular fibrillation, that was spontaneously reversible within 1 minute, also developed in 3 out of the 14 surviving control animals. One animal survived the reperfusion period without developing any arrhythmias. Neither SEA 0400, nor ORM 10962 pretreatments could significantly influence the development or severity of these arrhythmias, as well as the survival rate (Table 6 and Figure 10).

Table 6. Survival rate and incidence of ventricular arrhythmias during reperfusion after 6 min coronary artery occlusion in anesthetized rats

Reperfusion								
Groups	N	Survived		Incidence of arrhythmias (n/%)				Arrhythmia score
		n	%	None	VF	VT	VEB	
Control	21	14	67	1/5	10/48	13/62	8/38	4.00±0.4
SEA 0400	17	11	65	1/6	10/59	8/47	8/47	3.94±0.51
ORM 10962	25	17	68	0/0	15/60	14/56	14/56	4.12±0.35

N: total number of animals; n: number of the animals with the given response; %: percentage of the animals with the given response; None: animals without any arrhythmias; VF: ventricular fibrillation; VT: ventricular tachycardia; VEB: ventricular extrasystole and bigeminia.

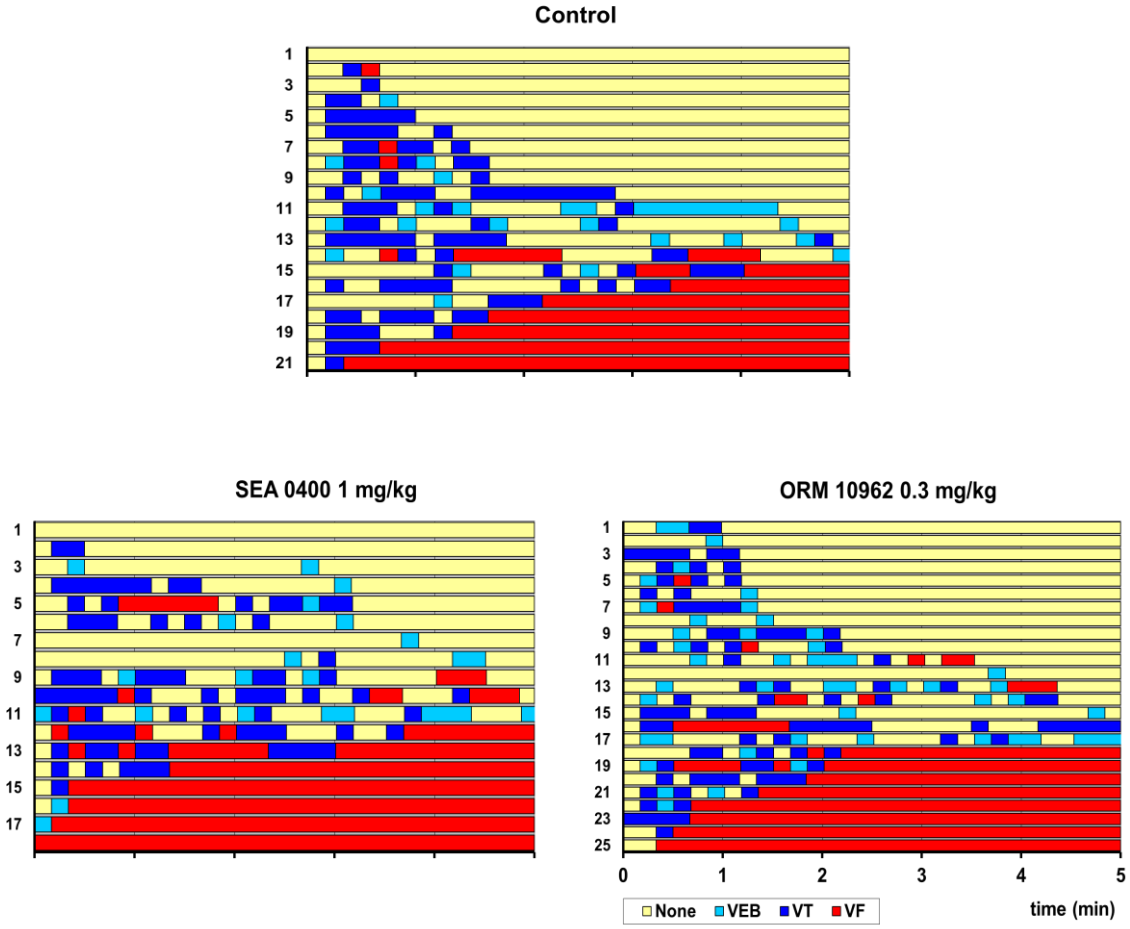


Figure 10. Arrhythmia map during reperfusion after 6 min coronary artery ligation in anesthetized (pentobarbitone, 60 mg/kg *i.p.*) rats after SEA 0400 (1 mg/kg) or ORM 10962 (0.3 mg/kg) pretreatment. The ordinates show the arrhythmia episodes of individual animals. None: animals without any arrhythmias; VF: ventricular fibrillation; VT: ventricular tachycardia; VEB: ventricular extrasystole and bigeminia.

6.2.2 Heart rate and blood pressure changes during coronary artery occlusion/reperfusion-induced arrhythmia model in rats

There were no significant differences in heart rate, arterial systolic- or diastolic blood pressure among the groups before the coronary artery occlusion. Coronary artery occlusion in the control group resulted in a modest elevation in the heart rate, as well as a modest decrease in the systolic- and diastolic blood pressure, which is maintained during the reperfusion, too. Neither SEA 0400, nor ORM 10962 pretreatments influenced significantly the heart rate and blood pressure response compared to the control (Table 7).

Table 7. Changes of the heart rate and blood pressure during ischaemia and reperfusion in rats

	Time of ischaemia (min)						Time of reperfusion (min)			
Group	n	-10	0	1	3	5	n	1	3	5
Control										
HR		427±9.0	410±9.0	415±10.3	418±10.9	415±11.4		416±10.9	407±13.1	417±13.4
SP	21	108±2.3	107±2.3	78±2.6	81±3.4	84±3.6	13	89±8.5	95±7.6	94±6.6
DP		71±3.4	71±3.4	44±2.0	48±2.6	53±3.3		55±7.7	61±7.9	59±7.0
SEA 0400										
HR		438±10.5	412±13.4	404±13.8	413±13.0	413±13.1		395±17.4	396±18.0	401±15.2
SP	18	114±2.7	111±3.2	87±3.9	92±4.3	96±5.0	11	89±5.8	100±7.0	101±8.8
DP		78±3.4	76±3.3	54±3.5	60±4.0	64±4.9		57±5.4	65±6.9	68±7.8
ORM 10962										
HR		411±8.5	397±8.2	410±9.6	404±9.4	401±10.7		387±14.5	400±10.1	384±15.0
SP	25	105±3.0	109±3.2	84±3.6	87±3.3	90±3.4	16	88±6.1	94±7.2	100±8.8
DP		73±3.3	77±3.7	54±2.8	58±2.7	61±3.0		56±5.2	64±6.9	69±7.5

n: number of the animals, HR: heart rate (beats/min), SP: systolic blood pressure (mmHg), DP: diastolic blood pressure (mmHg), -10 min: drug administration, 0 min: beginning of the occlusion. Results are expressed as mean ± S.E.

6.2.3 Effects of SEA 0400 and ORM 10962 pretreatments on ouabain-induced arrhythmias in guinea-pigs

Intravenous ouabain infusion induced various arrhythmias in guinea-pigs. The first arrhythmias, such as extrasystole and bigeminia, developed around the 24th minute in the control group, using an infusion rate of 10μg/kg/min ouabain. Later, ventricular tachycardia appeared which followed by ventricular fibrillation or AV block around the 37th minute after starting ouabain infusion (Figure 11).

Pretreatment with SEA 0400 or ORM 10962 significantly delayed the onset time of ventricular arrhythmias as compared to the control group. There were no significant differences between the two NCX inhibitor treated groups. Verapamil was also significantly delayed the onset time of ventricular extrasystole, bigeminia, tachycardia and fibrillation (Table 8).

Table 8. Onset time of ouabain-induced arrhythmias in guinea pigs

Group	Dose (mg/kg)	n	VEB (min)	VT (min)	VF/AVbl(min)
Control	-	21	24.0±1.73	31.8±1.85	36.9±1.81
SEA 0400	1.0	16	36.4±2.12 *	43.3±2.32 *	47.4±3.1 *
ORM 10962	0.3	11	36.6±2.64 *	40.8±2.17 *	42.4±2.1
Verapamil	0.32	7	48.0±4.06 *	51.4±3.23 *	58.0±2.8 *

n: number of animals in the group; VEB: ventricular extrasystole or bigeminia; VT: ventricular tachycardia; VF: ventricular fibrillation; AVbl: atrioventricular block. * $P<0.05$ vs. control. Results are mean ± S.E.

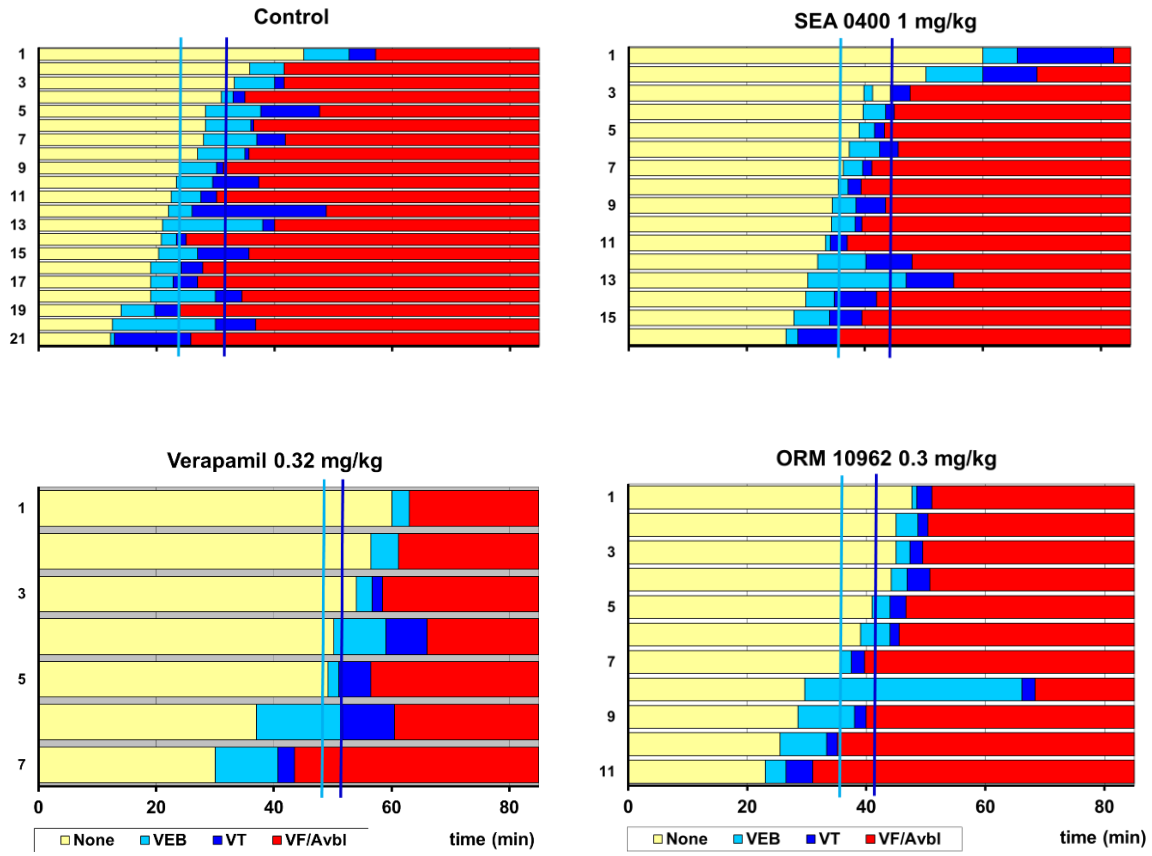


Figure 11. Arrhythmia map of ouabain-induced arrhythmias in guinea-pigs of the control, SEA 0400, verapamil and ORM 10962 pretreated groups.
None: no arrhythmia; VEB: ventricular extrasystole and bigeminia; VT: ventricular tachycardia; VF: ventricular fibrillation; AVbl: atrioventricular block. Each row represents a single animal and its "arrhythmia history". The vertical lines show the mean of the onset time of arrhythmias; light blue – VEB, dark blue – VT

6.2.4 Heart rate and blood pressure changes in ouabain-induced arrhythmia model

Due to continuous arrhythmias after 24 minutes (as a mean value) of ouabain administration, we followed the heart rate and blood pressure changes in the control group until the 19th minute. There were no significant differences in these parameters between the control, SEA 0400, ORM 10962 or verapamil treated animals before the drug administration (-10 min) (Figure 12). Verapamil pretreatment significantly decreased the heart rate and the systolic- and diastolic blood pressure 10 minutes after its administration. On the other hand, none of the NCX inhibitors influenced significantly the basal heart rate or blood pressure (Figure 12).

Ouabain increased the heart rate and the blood pressure during its infusion. All of the pretreatments (SEA 0400, ORM 10962 and verapamil) delayed the blood pressure response to ouabain in parallel with delaying the appearance of arrhythmias.

Hence, the last measured pressure values before the beginning of severe arrhythmias were at 30 and 40 minutes (Figure 11).

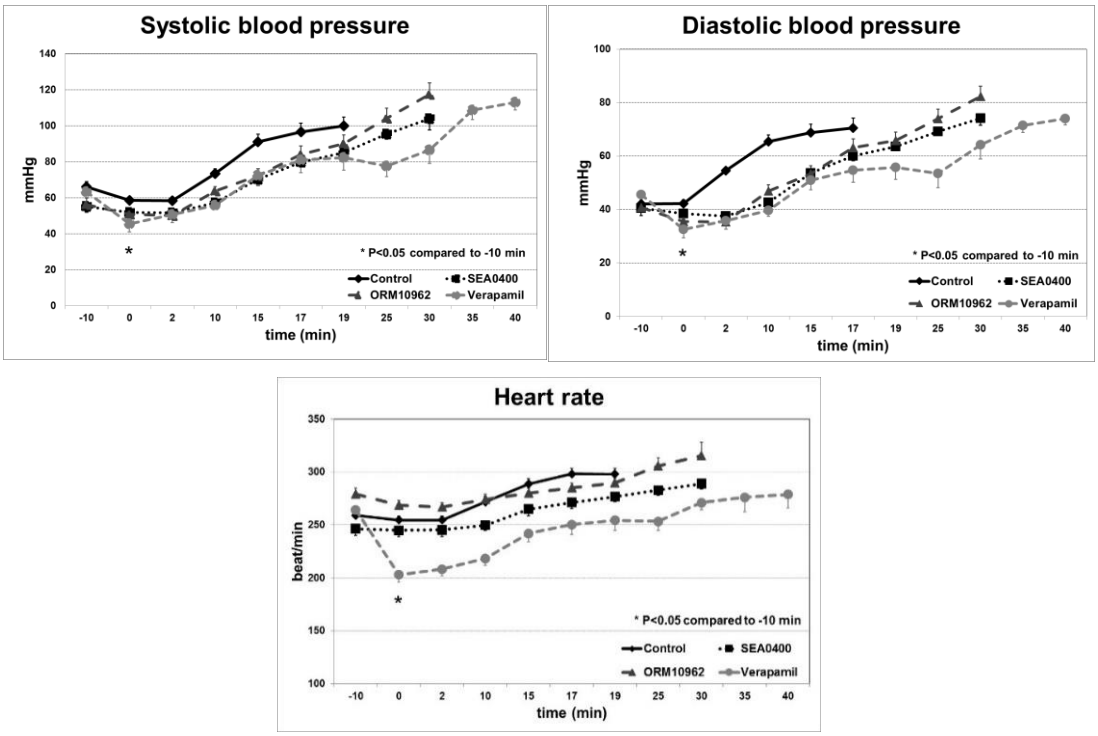


Figure 12. Systolic- and diastolic blood pressure, and heart rate changes in guinea pigs during ouabain infusion. Timescale: -10 min: vehicle, or drug administration (SEA 0400 1 mg/kg; ORM 10962 0.3 mg/kg, verapamil 0.32 mg/kg) 0 min: beginning of the ouabain infusion. For the number of animals see Table 8. Results were expressed as mean ± S.E.

6.2.5 Effects of ORM 10962 on the suspension of ouabain-induced arrhythmias

Intravenous administration of ouabain (10µg/kg/min) did not produce severe arrhythmias during the 16 min infusion. Termination of the infusion of ouabain at this time point, however, resulted in the progression of arrhythmias. In the control group, all of the animals developed some kind of arrhythmia; ventricular tachycardia developed in three animals, and even reversible ventricular fibrillation occurred in one animal (Figure 13).
Heart rate and blood pressure could not be determined during this period due to frequent arrhythmias. After ORM 10962 treatment, the progression of ouabain-induced arrhythmias was less expressed, only one animal developed ventricular tachycardia and no ventricular fibrillation occurred. The duration of the arrhythmic period was significantly shorter compared to the control group and the arrhythmias during this time were not continuous (Figure 13 and Table 9).

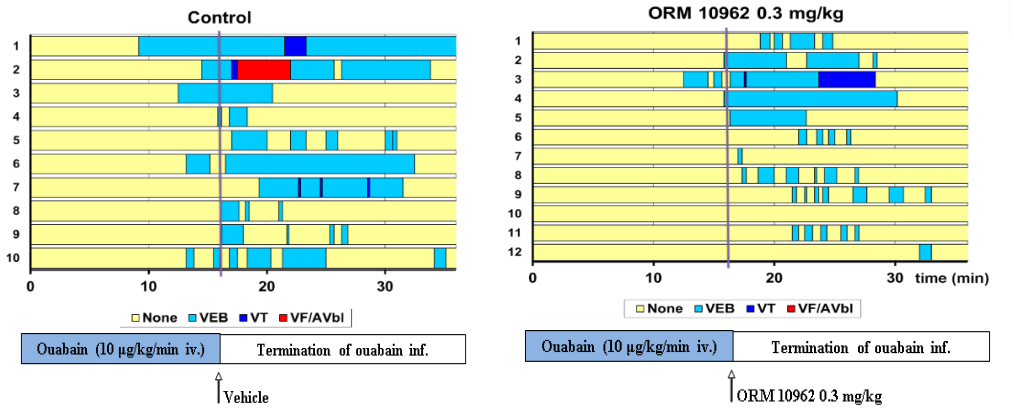


Figure 13. Arrhythmia maps of the suspension of ouabain-induced arrhythmias in guinea-pigs in the control or ORM 10962 treated groups. For details see Figure 11.

Table 9. Suspension of ouabain-induced arrhythmias by ORM 10962, administered after the termination of ouabain infusion (10 µg/kg/min for 16 min) in anesthetized guinea pigs

Group	Dose (mg/kg)	n	Arrhythmic period (min)	VEB (min)	VT (min)	VF/AVbl (min)
Control	-	10	14.22±2.29	10.5±2.5	0.3±0.2	0.5±0.5
ORM 10962	0.3	12	6.88±1.43 *	5.03±1.3	0.0	0.0

Note: n: number of animals in the group; Arrhythmic period: time between the appearance of the first and the termination of the last arrhythmic event; VEB: ventricular extrasystole and bigeminia; VT: ventricular tachycardia; VF/AVbl: ventricular fibrillation or complete atrioventricular block; * P<0.05

7. DISCUSSION

7.1 Antiarrhythmic effect of amiodarone and its stable metabolite, desethylamiodarone

The anesthetic agent (Baczkó et al., 1997), artificial ventilation and acute surgery may greatly and variably influence the development of arrhythmias during the acute phase of myocardial infarction (Leprán et al., 1983). Therefore, we used a conscious animal model where the acute phase of myocardial infarction develops in intact, freely moving animals.

The present results demonstrate that chronic oral DEA pretreatment (using half of the dose of AMIO) produced similar DEA levels in the plasma and myocardium as the double dose of AMIO. We also demonstrated that DEA – the main metabolite of AMIO – exerted similar antiarrhythmic effects to its parent compound, after long-term oral pretreatment in an acute coronary artery ligation-induced ventricular arrhythmia model in conscious rats.

AMIO treatment is still the most frequently used and effective therapy to prevent arrhythmias in the clinical practice. The advantages are its negligible negative inotropic effect and the very low incidence of proarrhythmia. On the other hand, the long-term routine use results in several problems due to the unique pharmacokinetic properties and its serious extracardiac adverse effects. During the first pass metabolism, AMIO is extensively and variably metabolized to DEA. The major enzymes responsible for this reaction in human beings are the cytochrome P450 1A1, 3A4 and 1A2 isoenzymes (Elsherbiny et al., 2008).

Both AMIO and DEA are further metabolized by dealkylation, hydroxylation and deamination (Ha et al., 2001). Enzyme polymorphism, the co-administration of enzyme inhibitors or inducers greatly influence the rate of conversion of amiodarone and it is responsible for the great individual variations in response and for its interaction with other drugs or food (Becquemont et al., 2007; Heimark et al., 1992; Libersa et al., 2000; Werner et al., 2004).

Previous investigations showed, that DEA – given as a single dose of 50 mg/kg intraperitoneally, 30 min before the coronary artery ligation in a similar arrhythmia model in conscious rats – significantly improved the survival (Varró et al., 1987). Our present investigations, using long-term oral pretreatment with DEA, supports and extends these earlier experiments. In the present experiments DEA (50mg/kg) offered significant protection against the early phase of arrhythmias, as well as produced similar plasma concentrations as it is measured after the double dose of AMIO (100 mg/kg) (Morvay et al., 2016). Therefore, the smaller dose of DEA may produce less adverse effects and less problem of drug interactions during its long-term use.

An amiodarone derivative, the non-iodinated dronedarone is also developed, and it is considered to have less adverse effects than amiodarone. Dronedarone is a less lipophilic, multichannel blocking drug, with lower tissue accumulation and much shorter serum half-life (approximately 24 hours). It has no harmful thyroid effects (containing no iodine); however, other gastrointestinal effects (nausea, vomiting and diarrhea) are often associated with its administration. Dronedarone seems to be effective at reducing ventricular tachycardia, recurrence of AF, and cardiovascular morbidity and mortality in patients with AF or atrial flutter. However, dronedarone was associated with increased mortality in patients having severe heart failure and left ventricular dysfunction (Brenner & Delacretaz, 2011; Garcia & Cheng-Lai, 2009; Oyetayo et al., 2010).

Dronedarone, similarly to amiodarone, prolongs the ventricular and atrial refractory period, prolongs the action potential duration, suppresses phase 4 depolarisation., and decreases the slope of the action potential upstroke (phase 0), which reflects blocking of fast sodium channel activity of the myocardium in rabbit and guinea-pig hearts (Gautier et al., 2003; Sun et al., 1999).

Investigations in isolated canine heart showed that the effect of long-term administration of dronedarone strikingly differs from that of long-term administration of amiodarone. Chronic dronedarone treatment does not produce considerable prolongation of the repolarization in dog ventricular muscle, as well as it does not affect the level of thyroid hormones, as opposed to amiodarone. Dronedarone thought to be less effective antiarrhythmic than amiodarone but also has less toxicity (Tadros et al., 2016).

Chronic administration of the main metabolite of dronedarone (N-debutyl dronedarone) does not exert electrophysiologically active plasma and cardiac tissue levels. This is partly the reason for the relatively short duration of the effect of dronedarone. On the other hand, DEA, the main metabolite of AMIO, accumulates in the cardiac tissue, hence DEA could be responsible for the difference in the chronic electrophysiological effects of dronedarone and amiodarone (Varró et al., 2001).

The accumulation of DEA is considered to be responsible also for the inhibition of the thyroid hormone metabolism and/or the blockade of the thyroid hormone receptors during chronic treatment with AMIO. Furthermore, the decrease of T3 by DEA may affect the potassium channel protein synthesis leading to a decreased density of different potassium channels (Kodama et al., 1996; Kodama et al., 1999; Varró et al., 1996), that may have a significant contribution to the antiarrhythmic effect.

7.2 Antiarrhythmic effects of sodium/calcium exchange inhibition

According to our investigations, selective NCX blockade by SEA 0400 and ORM 10962 proved to be effective against DAD related arrhythmogenesis in *in vivo* ouabain-induced cardiac arrhythmias in guinea-pigs but failed to exert cardioprotection against ischaemia/reperfusion-induced arrhythmias in rats.

The ouabain-induced arrhythmia model is widely used to investigate antiarrhythmic effects in different species e.g. guinea-pigs and dogs (Miyamoto et al., 2002; Tanaka et al., 2007; Watano, et al., 1999). Ouabain increases the arterial systolic- and diastolic blood pressure and has a positive inotropic effect via the inhibition of the Na^+/K^+-ATPase, resulting in the accumulation of intracellular Na^+ and thereby activating the reverse mode of NCX. Therefore, more Ca^{2+} is transported into the cell than out, which can be arrhythmogenic due to the Ca^{2+} overload (Imahashi et al., 1999; Lee & Hryshko, 2004; Padilha et al., 2008; Padilha et al., 2011).

According to our results, both SEA 0400 and ORM 10962 offered significant protection against ouabain-induced arrhythmias in guinea-pigs, demonstrated by the delay of the development of ventricular arrhythmias. The mechanism of the antiarrhythmic effect may be based on the inhibition of the increase in intracellular Ca^{2+} level due to ouabain via the inhibition of the reverse mode activity of NCX.

KB-R7943 and SEA 0400 are the most frequently investigated NCX blockers. Recently a much more selective compound, ORM 10103, has been developed. The NCX blocking selectivity and potency are in the following order: KB-R7943 < SEA 0400 < ORM 10103 (Kormos et al., 2014; Matsuda et al., 2001; Nagy et al., 2014; Tanaka et al., 2002). The low selectivity of KB-R7943 (10 μM) means that while inhibiting the NCX current by 80%, it simultaneously reduces many other ionic currents, e.g. Na^+, L-type Ca^{2+}, as well as the delayed rectifier K^+ currents. In contrast, SEA 0400 at a concentration of 1 μM, inhibits 70–80% of the NCX function, moderately influence the above-mentioned currents (4, 9 and 4% respectively) (Amran et al., 2004; Tanaka et al, 2002).

Recently, another novel NCX inhibitor, ORM 10103 was investigated *in vitro*. ORM 10103, even at 10-times higher concentration (10 μM) that needed to inhibit NCX current, has no detectable effect on other transmembrane currents, except for minimal suppression of I_{Kr} (Jost et al., 2013). It is significantly decreased the number of strophanthidin-induced spontaneous diastolic Ca^{2+} release events *in vitro*, however, produced only moderate antiarrhythmic effect during simulated ischaemia/reperfusion *in vitro* (Kormos et al., 2014;). These results were interpreted as a consequence of the inhibition of reverse mode NCX. ORM 10103 has not been tested in an in vivo arrhythmia model, because of the very low solubility in physiologic conditions.

ORM 10962 is a derivative of ORM 10103, showing higher activity and even better selectivity to inhibit NCX current, and can be administered in *in vivo* conditions, as well (Kohajda et al., 2016). According to our results, ORM 10962 delayed the development of ouabain-induced ventricular arrhythmias, e.g. extrasystole, tachycardia and fibrillation in anesthetized guinea-pigs, presumably due to the inhibition of the reverse mode of NCX. No significant difference was found between SEA 0400 and ORM 10962, although ORM 10962 was administered in lower dose, than SEA 0400 (0.3 mg/kg vs. 1 mg/kg).

In addition, ORM 10962 was effective also when it is applied after intracellular Ca^{2+} overload, when arrhythmias are already established. The antiarrhythmic effect in this case may be explained by the inhibition of the NCX forward mode, resulting in less depolarization and thereby eliminating DAD's in Purkinje fibres and suppressing arrhythmias in ouabain-induced guinea-pig model (Kohajda et al., 2016).

During myocardial ischaemia, anaerobic metabolism results in intracellular acidosis and ATP depletion, thus inhibiting the Na^+/K^+-ATPase and activating the Na^+/H^+ exchanger (NHE). Hence, the intracellular Na^+ increases, which activates the reverse-mode of NCX resulting in a Ca^{2+} overload, cell injury and myocardial stunning (Imahashi et al., 2005; Lee & Hryshko, 2004). Selective inhibition of the reverse mode of NCX theoretically may offer a potential therapeutic tool for cardioprotection against ischaemia/reperfusion injury (Akabas, 2004).

In the literature several papers support that NCX inhibitors improve the contractile recovery after ischaemia in isolated hearts (Magee et al., 2003; Namekata et al., 2005; Yoshiyama et al., 2004) or *in vivo* during coronary artery occlusion/reperfusion in rats or dogs (Takahashi et al., 2003; Takahashi et al., 2004). However only one paper supported the antiarrhythmic effect of SEA 0400 (Takahashi et al., 2003). ORM 10103, another highly selective NCX inhibitor, moderately decreased the incidence of ischaemia/reperfusion-induced arrhythmias in Langendorff-perfused rat hearts (Szepesi et al., 2015). Other studies reported no effect by NCX inhibitors (SEA 0400 and KB-R7943) in the prevention of arrhythmias *in vivo* (Lu et al., 1999; Miyamoto et al., 2002; Nagasawa, Zhu et al., 2005). Furthermore, in these studies SEA 0400 and KB-R7943 were used in such high concentration, which also may inhibit the I_{CaL} current making the interpretation of the data more difficult.

According to our results, neither SEA 0400, nor ORM 10962 pretreatments resulted in any significant protection on the incidence or severity of ischaemia/reperfusion-induced ventricular arrhythmias in rats *in vivo.*

The explanation of the failure of NCX inhibitors against ischaemia/reperfusion-induced arrhythmias may be the complexity of the development of arrhythmias in this case, involving several different mechanisms, e.g. reentry, spontaneous automacy, DADs, and NCX may not play important role in each mechanism (Carmeliet, 1999).

In addition, it is suggested, that the initiating step to develop ischaemic damage is the activation of Na^+/H^+ exchanger (NHE). NHE inhibition offered a protective effect against ischaemia/reperfusion injury (Imahashi et al., 1998; Imahashi et al., 1999; Maddaford & Pierce, 1997). The NHE blocker cariporide provided more powerful antiarrhythmic efficacy when applied simultaneously with an NCX inhibitor (Szepesi et al., 2015). The inhibition of NCX alone to limit Ca^{2+} overload probably does not play important role in ischemia/reperfusion-induced arrhythmias in anaesthetized rats (Lu et al., 1999). These results suggest that the prevention of the initial step, i.e. Na^+ overload may be more important for cardioprotection than the prevention of Ca^{2+} overload.

We may conclude that the protective effect of SEA 0400 or KB-R7943 in the previous investigations was mainly due to their lower selectivity, i.e. inhibiting Ca^{2+} current, and not to their NCX inhibitory effect.

7.3 Study limitations

Our investigations were performed in healthy, undiseased hearts. Arrhythmias frequently occur in patients with several cardiovascular risk factors and accompanied diseases (e.g. hyperlipidaemias, hypertension, diabetes during chronic myocardial ischemia, in heart failure, etc.), where calcium homeostasis and NCX function are altered compared to healthy human being or healthy animals. Furthermore, animal species (i.e. rats in the present investigations), may have different Ca^{2+}-handling mechanisms and may respond differently to NCX inhibition in ischemia/reperfusion-induced arrhythmias. Therefore, further investigations are needed using different animal models, and accompanied disease models to prove the potential therapeutic value of NCX inhibition in cardiac arrhythmogenesis.

8. CONCLUSIONS

Our results showed that DEA – the main metabolite of AMIO – exerted similar antiarrhythmic effects to its parent compound after long-term oral pretreatment in an acute coronary artery ligation-induced arrhythmia model in rats. We also demonstrated that chronic oral treatment with DEA resulted in similar cardiac tissue levels to that measured after chronic AMIO treatment. The adverse effects of chronic AMIO therapy may depend on its tissue accumulation in various organs. It is expected that substituting DEA for AMIO in the clinical practice would result in a better therapeutic option, resulting similar effectiveness with less toxicity. Using the active metabolite of AMIO, i.e. DEA, we may exclude individual variations in the metabolism of AMIO to DEA, also decrease the interaction of this metabolic reaction with other drugs and food, leading to an easier dosage optimalization and less adverse effects.

Selective inhibition of Na^+/Ca^{2+} exchanger by ORM 10962 or by SEA 0400 offers significant protection against ouabain-induced arrhythmias in guinea-pigs. The more selective ORM 10962 exerts the same protective effect in smaller dosage compared to SEA 0400.

On the other hand, ORM 10962, a highly selective NCX inhibitor, did not influence myocardial ischaemia/reperfusion-induced arrhythmias in anesthetized rats. This uneffectiveness may be due to the secondary role of NCX in the development of arrhythmias under ischaemia/reperfusion conditions. The controversy to the literature findings, that some NCX inhibitors found to be antiarrhythmic in such conditions, may be explained by the less selectivity of former NCX inhibitors, i.e. inhibiting also voltage-dependent calcium channels in the applied doses.

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11. ANNEX

I.

II.

12. CO-AUTHOR CERTIFICATION

Társszerzői lemondó nyilatkozat

Alulírott, Prof. Dr. Varró András – mint felelős társszerző – kijelentem, hogy Farkas-Morvay Nikolett (pályázó) PhD értekezésének tézispontjaiban bemutatott - közösen publikált – in vivo vizsgálatok eredményei saját, önálló munkája, eredményeit felhasználhatja PhD értekezéséhez. Ezeket a téziseket más a PhD fokozat megszerzését célzó minősítési eljárásban nem használta fel, illetve nem kívánja felhasználni.

Szeged, 2019.06.05. …….....

dátum szerző

A pályázó tézispontjaiban érintett, közösen publikált közlemények:

The effect of a novel highly selective inhibitor of the sodium/calcium exchanger (NCX) on cardiac arrhythmias in *in vitro* and *in vivo* experiments.

*Kohajda Z, **Farkas-Morvay N**, Jost N, Nagy N, Geramipour A, Horváth A, Varga RS, Hornyik T, Corici C, Acsai K, Horváth B, Prorok J, Ördög B, Déri S, Tóth D, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Tóth A, Baczkó I, Leprán I, Nánási PP, Papp JG, Varró A, Virág L. PLoS One. 2016 Nov 10;11(11):e0166041.*