Ph.D. THESIS

The effect of SEA0400 on the calcium handling of mammalian ventricular myocytes

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DEBRECEN, 2008.

I. INTRODUCTION

I/1. The calcium handling of cardiomyocytes

The rythmic functioning of the heart is established by the action potencials forming in the sinusnode and spreading through the stimulus conducting system, and then spreading through the workmuscle. As a result of the change in the membrane potential of the cell intracellular calcium concentration ($[Ca^{2+}]_i$) is temporarly elevated (it is called $[Ca^{2+}]$ trasient) which activates the contractile system. Three mechanisms participate in the increase of $[Ca^{2+}]_i$, i.e. in the development $[Ca^{2+}]_i$ transients. The Ca^{2+} entering through the L-type Ca^{2+} channels of the cell membrane from extracellular matrix is responsible for the smaller part of the increase of $[Ca^{2+}]_i$. The greater part of $[Ca^{2+}]_i$ increase is released from sarcoplasmic reticulum (SR) through the Ca^{2+} channels in the SR membrane, and through ryanodin receptors (RyR2). The third source of calcium ions getting into intracellular space is the Na^+/Ca^{2+} exchanger (NCX), which increases Ca^{2+} in the initial phase of action potential, while it is pumping Na^+ out of the cell. At this phase the membrane potential is positive and the level of intracellular Ca^{2+} is relatively low. A part of Ca^{2+} liberated within the cell is contacted to special Ca^{2+}-bind proteins, the conformation change of which transmits between the increase of the level of Ca^{2+} in the cell and physiological function. The contraction is regulated by the troponin complex on the thin filament which enables the connection between thin and thick filaments after connecting Ca^{2+} and thus it enables the shortening cardiomyocytes. The energy depending procedure closes, when the level of Ca^{2+} recovers to resting value before contraction. The relaxation of the Ca^{2+} transient is the result of three competitively functioning mechanism. At least to thirds of the liberated Ca^{2+} in the cell plasm is taken up by the SR with the help of Ca^{2+}-ATPase (SERCA). Depending on the species the NCX extrudes 7-30% of the liberated Ca^{2+} content of the cellplasm and only a small quantity leaves through the slow Ca^{2+}-transport systems: through sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+}-transport.

I/2. The role of NCX in calcium handling

The Ca^{2+} balance of the cell requires that in steady-state equilibrium, the beat-to-beat Ca^{2+} influx and efflux must be equal in the wide range of physiological conditions to avoid Ca^{2+} loss or Ca^{2+} overload in the cell, which means that the main transmembrane Ca^{2+} fluxes in both directions need to be finely regulated.

The Na^+/Ca^{2+} exchanger is a countertransport mechanism located in the cell membrane of almost every mammalian cell type. In heart muscle it was identified about forty years ago by measuring the dependence of Ca^{2+} efflux from cardiac muscle on ionic composition. Later it was determined that it exchanges 3 Na^+ for 1 Ca^{2+}, which implies that its operation is electrogenic, i.e. results in electrical current flowing across the membrane. The direction of this current corresponds to the mode of operation of the Na^+/Ca^{2+} exchanger. In forward mode operation (Ca^{2+} efflux), an inward depolarizing current flows into the cell, while the reverse mode (Ca^{2+} influx) results in a concomitant outward current. Resulting partly from its electrogenic property, activity of the Na^+/Ca^{2+} exchanger has a strong dependence on the instantaneous value of the membrane potential. Due to this and to the strong dependence of the Na^+/Ca^{2+} exchanger activity on the electrochemical gradients of the transported ions, operation of the Na^+/Ca^{2+} exchanger follows a complicated time course during the cardiac cycle. In addition, besides influencing the thermodynamic drivingforces, it has been shown that intracellular Na^+ and Ca^{2+} also regulate the Na^+/Ca^{2+} exchanger in an allosteric manner, making the understanding of its in situ regulation and activity exceptionally difficult. So the NCX–partly due to the regulation of the Ca^{2+} content of the cell and partly due to the ioncurrent generated during its functioning – plays a major role in arrhythmogenesis, so as a result its selectiv blocking plays a key role in the therapy of arrhythmia.

I/3. The role of NCX in arrhythmogenesis

The two most important biochemical changes after myocardiac ischaemia are acidosis developing as a result of the anaerob function and ATP depletion developing from decreased ATP production. Another consequence of ATP depletion is the decreased functioning of Na^+/K^+ pump, which leads to the accumulation of intracellular Na^+ which aggravated by Na^+ influx developing through NCX. The increased intracellular Na^+ concentration leads to Ca^{2+} elimination and the increased influx of Ca^{2+} via NCX. In cardiomyocytes surviving ischaemia the increased

intracellular Ca^{2+} concentration can lead to cummulated arrhythmia during early reperfusion. The development of Ca^{2+}-accumulation and the generation of arrhythmias as a result can be blocked by the selective inhibition of NCX. By blocking NCX we can moderate both early and delay type of afterdepolarization. As a result of forward mode blocking the inward current will be smaller, which induces afterdepolarizational episodes, while reverse mode blocking decreases the quantity of Ca^{2+} entering the cell.

Digitalis derivaties as drugs with a positive inotrop effect, exert their influence especially via the blocking of Na^+/K^+ ATPase in a way that the NCX transmits Na^+ is transmited outward, while Ca^{2+} is transmitted to the myocytes in the intracellular space due to the effect of increased Na^+. The increased Ca^{2+} concentration in the intracellular space will induce a stronger contraction force. During digitalis intoxication the myocytes are overloaded with Ca^{2+}, which induces arrhythmia. We can eliminate digitalis-arrhythmia by reverse mode NCX blocking, because this way we can prevent the overloading of the myocytes with Ca^{2+} and the development conquesential after depolarization.

I/4. Pharmacology of the NCX blocker

An important way to examine the role of a transport system in physiological or pathological situations is to block the transporter with a selective inhibitor. Until recently, however, this possibility was hampered in case of Na^+/Ca^{2+} exchanger because of the lack of potent and highly specific inhibitors. Therefore, although there are several pharmacological agents inhibiting the Na^+/Ca^{2+} exchanger, the interpretation of results obtained using these compounds is complicated by the concomitant effects on other transport systems or ionic channels. For example, amiloride analogues have been used to study Ca^{2+} homeostasis in cardiac preparations, but these agents have also been shown to block the Na^+/H^+ exchanger, making the interpretation of their effects regarding the role of Na^+/Ca^{2+} exchanger in Ca^{2+} handling rather difficult. Several trivalent and divalent cations are also capable to inhibit the Na^+/Ca^{2+} exchanger, among which Ni^{2+} has been used extensively to identify the Na^+/Ca^{2+} exchanger current in electrophysiological experiments. The main advantage of using Ni^{2+} is that it blocks Na^+/Ca^{2+} exchanger totally in a reversible manner. In general, these nonselective molecules and ions can only be used in subcellular systems or in experiments in which the Na^+/Ca^{2+} exchanger is

isolated by means of applying other blockers and ionic substitutions.

Therefore, the newly developed potent and selective Na^+/Ca^{2+} exchanger inhibitors present a great advance in the field of the Na^+/Ca^{2+} exchanger research, as by using these molecules the role of the Na^+/Ca^{2+} exchanger can be investigated in intact cells or tissues under physiological circumstances. The two most selective and widely used Na^+/Ca^{2+} exchanger inhibitors in the literature are the aniline derivative SEA0400 (2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxy-aniline) and the isothiourea derivative KB-R7943 (2-[2-[4-94-nitrobenzyloxy)-phenyl]ethyl]-isothiourea). When examining the role of the Na^+/Ca^{2+} exchanger in physiological as well as pathological circumstances by using pharmacological tools, it is important to know the selectivity, potency and possible direction dependence of the inhibitors.

An early study investigating the pharmacological effect of KB-R7943 concluded that this compound affects the Na^+/Ca^{2+} exchanger in a Ca^{2+}-dependent manner under certain experimental circumstances, suggesting that an interaction might exist between the drug and the intrinsic regulatory mechanisms of the Na^+/Ca^{2+} exchanger. Similar interaction has been suggested to be involved in the mechanism of action of SEA0400, as its blocking effect was found to be dependent on the intracellular Na^+ concentration, and related inversely to the intracellular Ca^{2+} concentration. However, these experiments were carried out in an expressed system using non-physiological ionic concentrations to measure the Na^+/Ca^{2+} exchanger current.

In the recent years, several studies indicated that inhibition of the Na^+/Ca^{2+} exchanger can be beneficial in experimental models of cardiac disorders. Most of these studies examined the involvement of the Na^+/Ca^{2+} exchanger in the ischemic damage of the myocardium. Based on the thermodynamical properties of the operation of the Na^+/Ca^{2+} exchanger, intracellular accumulation of Na^+ in ischemic/reperfused myocardium favors the reverse mode operation of the Na^+/Ca^{2+} exchanger, which may contribute to the intracellular accumulation of Ca^{2+} leading to cell damage and death. Therefore, it can be speculated that pharmacological inhibition of the Na^+/Ca^{2+} exchanger may display cardioprotective effects under such circumstances. In accordance with this hypothesis, it has been reported that SEA0400 reduced infarct size in rat and rabbit hearts after ischemia/reperfusion. The beneficial effects exerted by the Na^+/Ca^{2+} exchanger inhibitors in ischemia/reperfusion were also demonstrated in large animal models such as dog

and pig. The above and other studies also examined the proposed mechanisms of the observed protective effects of the Na^+/Ca^{2+} exchanger inhibitors in such circumstances. Indeed, it has been verified that the most likely mechanism of action was the prevention of the excessive Ca^{2+} overload by inhibiting the reverse mode operation of Na^+/Ca^{2+} exchanger. The possible therapeutic potential of Na^+/Ca^{2+} exchanger inhibitors in ischemia/reperfusion injury was recently reviewed.

Forward mode operation of Na^+/Ca^{2+} exchanger may also be related to pathological events, like early and delayed afterdepolarizations, in the heart when the intracellular Ca^{2+} concentration is elevated. In forward mode (Ca^{2+} efflux) operation, an inward depolarizing current flows into the cell, which, when the intracellular Ca^{2+} level is elevated, can be strong enough to induce afterdepolarizations leading to severe cardiac arrhythmias. Supporting this idea, a suppressive effect of SEA0400 on digitalis-induced arrhythmias was observed in canine models. We also found that SEA0400 effectively decreased the occurrence of early and delayed afterdepolarizations in canine cardiac tissues. The role of Na^+/Ca^{2+} exchanger in such circumstances and the possible antiarrhythmic potential of its inhibitors were reviewed.

According to studies it seems that SEA0400 is an extremly effective NCX blocker, which is clinically promising and probably posseses great selectivity. So it was our objective to investigate the effects of the SEA0400 on the Ca^{2+} handling of myocytes, the parameters of inracellular Ca^{2+} transients and contractile answers on preparates (canine and guinea pig), the action potential of which can be compared to the lengh of human plato phase. We thought it important because the heart function of murines (rats, mices) with small bodies and mammalians with bigger bodies display significant differences both from the aspect electrophysiology and the aspect of Ca^{2+} handling. Primarirly, in our experiments we used canine heart beacuse on the basis of its electrophysiological properties (action potential and transmembrane ion-currents) it is the most similar to the human heart.

II. OBJECTIVES

The aim of our experiments was the detailed analysis of the effect of NCX inhibitor SEA0400 on ioncurrents and the Ca^{2+} handling of myocytes on canine and guinea pig preparates. Within this range we tried to answer the following concrete questions:

1. How does SEA0400 effect the amplitude of intracellular Ca^{2+} transient of canine ventricular myocytes, diastolic and sistolic Ca^{2+} concentrations and cell-shortening?
2. How does SEA0400 effect the release of Ca^{2+} from SR vesicules isolated from canine heart, and the uptake of Ca^{2+} of the vesicules?
3. Does SEA0400 modify the gating of isolated RyR2 channels incorporated in the lipid bilayer?
4. Does SEA0400 effect the Ca^{2+} sensitivity of contractile proteins?
5. Beside the NCX blocking effect of SEA0400 does it modify the other important factor of Ca^{2+} handling, the quantity of Ca^{2+} influx through L-type Ca^{2+} channels?
6. What further ioncurrents can SEA0400 inhibit on canine ventricular myocytes, i.e. to what extent can we judge the NCX-blocking effect of SEA0400 selective?
7. Can we compare the effect of SEA0400 on Ca^{2+} handling and contractility between ventricular myocytes isolated from canine and Langendorff-perfused guinea pig heart?

Answering these questions we can judge if SEA0400 is a clinically promising drug and if it is the selective blocker of NCX.

III. MATERIALS AND METHODS

III/1. Electrophysiological examinations

III/1.1. Cell isolation

Isolated canine ventricular myocytes were obtained from hearts of adult mongrel dogs using the segment perfusion technique. The animals were anaesthetized and their hearts were rapidly removed from the chest. One of the coronary arteries (usually LAD) was cannulated and perfused with calcium-free JMM solution for 5 min in order to remove calcium from the tissues. Dispersion of the cells was performed during a 30 min perfusion with JMM solution containing 50 µM $CaCl_2$ and 1 mg/ml collagenase type CLS2. Cells were stored in MEM solution at 15 ^oC before we uses them for experiments.

III/1.2. Measurement of NCX current in voltage clamped myocytes

After establishing the whole cell configuration in Tyrode solution the cell was superfused with a special K^+-free bath solution supplemented with 20 µM ouabain, 1 µM nisoldipine and 50 µM lidocaine in order to block Na^+, K^+, Ca^{2+} and Na/K pump currents. NCX current was recorded using ramp pulses (having speed of 100 mV/s) delivered at a rate of 0.05 Hz. The membrane was initially depolarized from the holding potential of –40 mV to +60 mV, then hyperpolarized to -100 mV, and finally the membrane potential returned to the holding potential. Outward and inward NCX currents were determined during the descending limb of the ramp at +40 and –80 mV. After taking the control record in K^+-free solution, the cell was superfused with 1 µM SEA0400, and finally 10 mM $NiCl_2$ was added in order to fully block the current. Thus, total NCX current was determined at both membrane potentials as a Ni^{2+}-sensitive current by subtracting the third record from the first one. The fraction of block induced by SEA0400 was expressed as percent of total NCX current.

III/1.3. Measurement of $I_{Ca.L}$

$I_{Ca.L}$ was recorded from viable myocytes sedimented in a plexiglass chamber and continuously superfused with oxygenated Tyrode solution at 37 ^oC. I_{to} was suppressed by 3 mM 4-aminopyridine added to Tyrode solution. $I_{Ca.L}$ was recorded with an Axopatch-2B amplifier (Axon Instruments) using the whole cell configuration of the patch clamp technique. After establishing high (1-10 GΩ) resistance seal by

gentle suction, the cell membrane beneath the tip of the electrode was disrupted by further suction or by applying 1.5 V electrical pulses for 1-5 ms. After this step, the intracellular solution was allowed to equilibrate with the pipette solution for a period of 5-10 min before starting the measurement.

$I_{Ca.L}$ was elicited by 400 ms long depolarizations to +10 mV arising from the holding potential of –40 mV. These depolarizations were delivered to the preparations at a frequency of 0.2 Hz. Peak current density was defined as a difference between the peak value of $I_{Ca.L}$ and its pedestal measured at the end of the pulse. $I_{Ca.L}$ was normalized to cell capacitance, determined in each cell using short (25 ms) hyperpolarizing pulses from 0 mV to –10 mV. Experiments were discarded when the series resistance was substantially increasing during the measurement, or when the amplitude $I_{Ca.L}$ declined during the equilibration period.

III/1. 4. Recording of single channel currents of canine ryanodine receptor (RyR2) incorporated into artificial lipid bilayer

Heavy sarcoplasmic reticulum (SR) vesicles were isolated from ventricular free wall of dogs, and the solubilized RyR2 was purified. Single channel bilayer measurements were carried out using solubilized RyR2 incorporated into planar lipid bilayer. Bilayers were formed across a 250 μm aperture of a nolrene cap using symmetrical buffer solution. The chamber into which the small aliquot of the solubilized RyR2 has been added was designated as the *cis* (cytoplasmic) side, while the other chamber was labelled as *trans* (luminal) side and was kept on ground potential. Current signals, obtained under voltage clamp conditions, were filtered at 1 kHz using an 8 pole low pass Bessel filter and digitized at 3.3 kHz using Axopatch 200 amplifier and pClamp 6.02 software. Channels having conductance higher than 400 pS were considered as RyR2 in the presence of 250 mM K^+ used as current carrier. Single channel measurements were carried out at 23 ^oC, free Ca^{2+} concentrations at both *cis* and *trans* sides were calculated to be 50 μM using the computer program and stability constants published by Fabiato.

III/2. Measurment of Ca^{2+} handling and contractility

III/2.1. Recording of left ventricular pressure and $[Ca^{2+}]_i$ transients in Langendorff-perfused guinea pig heart

Male guinea pigs (weighing 300-500 g) were anesthetized and their heart was rapidly removed and fixed to the cannula of a Langendorff-perfusion device. The heart was perfused with Krebs solution. The coronary perfusion was maintained at 37 ^{o}C using a peristaltic pump. Left ventricular pressure was continuously monitored using a Braun 2021-02 arterial pressure transducer that was connected to the left ventricular cavity.

To record $[Ca^{2+}]_i$ transients, the heart was loaded with the acetoxymethylester of the fluorescent dye, Fura-2 (5 mM). The dye was excited at both 340 and 380 nm wavelengths. The emitted light was collected at 510 nm using a trifurcated quartz fiber optic bundle connected to a Deltascan device. $[Ca^{2+}]_i$ was calculated from the background-corrected fluorescent ratio (F_{340}/F_{380}) signals. The analogue fluorescence and pressure signals were sampled at 1 kHz. In each case 10 subsequent beats were averaged and stored for later analysis.

III/2.2. Recording of $[Ca^{2+}]_i$ transients and cell shortening in field stimulated myocytes

Single canine myocytes were obtained from hearts of adult mongrel dogs of either sex. The cells were incubated with 2 µM Fura-2-AM (Molecular Probes, Eugene, OR, USA) for 30 min. After this incubation period the cells were superfused with normal Tyrode solution at 35 C. Myocytes were stimulated using an electronic stimulator (DS-R1, Fonixcomp Ltd, Hungary) at a constant frequency of 1 Hz through a pair of platinum electrodes. The chamber was attached to the stage of an inverted fluorescent microscope (IX71, Olympus, Japan). Cells were excited at 360 and 380 nm from a xenon arc lamp (Optosource, Cairn, UK). The excitatory wavelengths were selected using a galvanometric monochromator (Optoscan, Cairn, UK) at 100 kHz switch rate. The emitted light was band pass filtered and detected by a photomultiplier tube. The demultiplexed optical signals were recorded and analyzed using the Acquisition Engine software (Cairn, UK). Changes in $[Ca^{2+}]_i$ were characterized by the ratio of the fluorescence intensities obtained at 360 and 380 nm excitation (F_{360}/F_{380}) following corrections for nonspecific background and bleaching. Cell shortening was recorded using a video edge detector system (VED-105, Crescent Electronics, Sandy, Utah, USA) and expressed as percent of the diastolic

cell length.

III/2.3. Calcium flux measurements in SR membrane vesicles

Heavy SR (HSR) vesicles and the ryanodine receptor Ca^{2+} release channel (RyR2) were isolated from canine left ventricular myocardium. The vesicles were actively loaded with Ca^{2+}, and the Ca^{2+} efflux was determined by measuring the extravesicular Ca^{2+} concentration using a Fluoromax spectrofluorometer (Jobin-Yvon, New Jersey, USA) modified for absorption measurements by monitoring the transmittance at 710 and 790 nm and calculating the corrected absorbance change (A_{710} - A_{790}). In brief, vesicles were suspended in a medium containing 92.5 mM KCl, 1 mM $MgCl_2$, 180 µM antipyrylazo III (ICN Biomedicals, Aurora, OH, USA), 54 µM $CaCl_2$, and 18.5 mM K-MOPS, having a final protein concentration up to 260 µg/ml. Vesicles were actively loaded with Ca^{2+} in the cuvette by the addition of the appropriate amount of $CaCl_2$ and the uptake was initiated by the addition of 1 mM ATP.

The effect of SEA0400 on SR Ca^{2+} release was assessed in two ways. After completion of the ATP-ADP conversion a second Ca^{2+} injection was applied in order to activate the release channel by adjusting the extravesicular Ca^{2+} to 20 µM. SEA0400 was applied following stabilization of the rate of Ca^{2+} efflux. The rate of Ca^{2+} release was measured and compared before and after the addition of SEA0400. The Ca^{2+} injection contained much less Ca^{2+} setting extravesicular Ca^{2+} concentration to 2 µM only, so the rate of baseline Ca^{2+} efflux was very low. Then SEA0400 was added to see if there is an increase in Ca^{2+} release. Rate of Ca^{2+} efflux was calculated from the rate of change of the light intensity just prior to and after the application of SEA0400. At the end of the experiment Ca^{2+} ionophore (2 µM A23187) was used to check Ca^{2+} load. Extravesicular Ca^{2+} concentration was calibrated using a very similar protocol, in the same medium without the addition of ATP, and in the presence of 2 µM A23187. Calibration curve was obtained by stepwise elevation of Ca^{2+} while the A_{710}-A_{790} values were recorded. Free Ca^{2+} concentrations were calculated using the absolute stability constants and the computer program developed by Fabiato.

Effect of SEA0400 on the initial rate of Ca^{2+} uptake was studied in light SR (LSR) vesicles using a similar protocol, but in this case the vesicles were first incubated with a given concentration (0, 0.3, 1, or 3 µM) of SEA0400 for 15 min, then

the Ca^{2+} uptake was initiated by the addition of ATP and subsequently Ca^{2+}. The rate of Ca^{2+} uptake was determined by linear regression from the slope of light intensity changes using the time window between 30 and 150 s following the addition of Ca^{2+}.

III/2.4. Measurement of contractility in chemically skinned single myocyte preparations

Small frozen tissue blocks were first defrosted and mechanically disrupted in cell isolation solution. The suspension was incubated for 5 min in this solution supplemented with 0.3% Triton X-100 (Sigma, St. Louis, MO, USA), washed and kept in cell isolation solution on ice for a maximum of 12 hours. Subsequently, a demembranated single cardiomyocyte was mounted between two thin needles with silicone adhesive (Dow Corning, Midland, USA) while viewed under an inverted microscope (Axiovert 135, Zeiss, Germany). One needle was attached to a force transducer element (SensoNor, Horten, Norway) and the other to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). Measurements were performed at 15 ^{o}C, and the average sarcomere length was adjusted to 2.2 µm.

Composition of relaxing and activating solutions used in force measurements was calculated. The pCa values (i.e. -log[Ca^{2+}]) of the relaxing and activating solutions were 9.0 and 4.75, respectively. Solutions with intermediate Ca^{2+} levels were obtained by mixing activating and relaxing solutions. Isometric force was measured after the preparation had been transferred from the relaxing solution to a Ca^{2+}-containing one. When a steady level of force was reached, the length of the myocyte was abruptly (within 2 ms) reduced by 20% and then quickly restretched. As a result, the force first dropped from the peak isometric level to zero and then started to redevelop. The passive component of force was determined in relaxing solution following the Ca^{2+} contractures. Ca^{2+}-activated isometric force was calculated by subtracting the passive force from the peak isometric force, obtained at pCa=4.75. After the first maximal activation at pCa=4.75, the resting sarcomere length was readjusted to 2.2 µm, when it was necessary. This second maximal activation at pCa=4.75 was used to calculate maximal isometric force (P_{max}). Cells were subsequently exposed to a series of solutions with intermediate pCa values. The Ca^{2+}-activated force measured at these intermediate levels of activation was normalized to P_{max} and data were fit to the Hill equation in order to estimate Ca^{2+}

concentration resulting in half maximal force production (pCa_{50}). If at the end of this series, reexposure to pCa 4.75 yielded a P_{max} less than 80% of the initial value, the measurement was discarded.

III/3. Statistics

The arithmetic means and the standard error of mean were calculated from our results. Statistical significance was determined using one-way ANOVA followed by Bonferoni test. Differences were considered significant when the P value was less than 0.05.

IV. RESULTS

IV/1 . Effects of SEA0400 in Langendorff-perfused guinea pig hearts

The effect of 25 min consecutive perfusion with 0.3 and 1 µM of SEA0400 was studied in 6 Langendorff-perfused guinea pig hearts. SEA0400 had no obvious effect on the left ventricular pressure in these preparations. No significant change was observed in the systolic pressure (55.5±2.7 mmHg in control *versus* the 58.6±3.5 and 53.0±3.9 mmHg measured at the end of 0.3 and 1 µM SEA0400 perfusion, respectively), end-diastolic pressure (6.0±0.2 mmHg *vs.* 6.8±0.4 and 6.4±0.4 mmHg), and pulse pressure (49.5±2.8 mmHg *vs.* 51.8±3.5 and 46.6±3.8 mmHg). The fluorescent ratio, used as indicator of $[Ca^{2+}]_i$, was also unchanged by the SEA0400 perfusion. The respective values for the peak fluorescent ratio were 1.04±0.01 *vs.* 1.04±0.02 and 1.05±0.03, for the baseline fluorescent ratio: 0.81±0.04 *vs.* 0.81±0.04 and 0.83±0.04, and for fluorescent ratio amplitude values of 0.23±0.03 *vs.* 0.22±0.03 and 0.22±0.03 were obtained.

Kinetic properties of pressure changes were characterized by time required to develop peak pressure (TtP), time required to achieve half relaxation (HRT), and by the maximum and minimum values of first time derivatives of the pressure (+dP/dt, -dP/dt) obtained during systole and diastole, respectively. No significant changes were observed in any of these parameters. The $[Ca^{2+}]_i$ transients obtained before and after SEA0400 treatment were analyzed in a similar manner, except for a segment of the decaying phase of the curve (from 30% to 90% of relaxation) which was fitted to a monoexponential function. SEA0400 caused no significant change in the TtP and

HRT values obtained for the fluorescent ratio, however, the decay time constant was significantly increased by 0.3 and 1 µM SEA0400 (from 127±7 ms to 165±7 and 177±14 ms, respectively, $P<0.05$).

IV/2. Effect of SEA0400 on $[Ca^{2+}]_i$ transient and cell shortening in field stimulated myocytes

Myocytes were stimulated at a constant frequency of 1 Hz through a pair of platinum electrodes. After establishing steady-state contractions and $[Ca^{2+}]_i$ transients, the perfusion was switched to the solution containing 0.1, 0.3, or 1 µM SEA0400, and 5 min was allowed to develop full drug effects. Data indicate that no significant change in these parameters was caused by SEA0400 in the 0.1-1 µM concentration range. Similarly, no significant change was observed in the decay time constant of the $[Ca^{2+}]_i$ transient determined before and after the application of 1 µM SEA0400 (269±41 and 266±17 ms, respectively, n=9).

IV/3. Effect of SEA0400 on SR calcium release

In HSR vesicle preparations the effect of SEA0400 was studied by measuring Ca^{2+} release from the vesicles in the presence of 20 µM extravesicular Ca^{2+}. This Ca^{2+} concentration makes the RyR2 partially open as seen from the increase of extravesicular Ca^{2+} concentration. Addition of SEA0400 failed to alter the rate of release as revealed by the unchanged slope of the Ca^{2+} flux, indicating that the drug did not modify Ca^{2+} flux through the partially open channel. The rate of Ca^{2+} release was 12.4±0.9 nmoles/min/mg protein before the application of SEA0400, while 11.8±1.1, 13.1±1.2, and 13.2±1.2 nmoles/min/mg protein in the presence of 0.3, 1, and 3 µM SEA0400, respectively (n=7 for each).

Similarly designed experiments were performed to study the effect of SEA0400 on closed RyR2 channels. In this case lower, just subthreshold extravesicular Ca^{2+} concentrations were applied. The horizontal segment of the record indicates that the channels are practically closed. SEA0400 failed to induce any Ca^{2+} release, in contrast to the Ca^{2+} ionophore, A23187 (2 µM) which caused marked Ca^{2+} release showing that the vesicles were indeed fully packed with Ca^{2+}. Under these experimental conditions the rate of baseline Ca^{2+} release was 2.2±0.31 nmoles/min/mg protein before the application of SEA0400, while 2.2±0.3, 2.3±0.28, and 2.3±0.3 nmoles/min/mg protein in the presence of 0.3, 1, and 3 µM

SEA0400, respectively (n=6 for each).

IV/4. Effect of SEA0400 on SR calcium uptake

Activity of the SR calcium pump was estimated by measuring the initial uptake rate of LSR vesicles by measuring time-dependent changes in extravesicular Ca^{2+} concentration under maximal pumping rate conditions. The uptake rate was determined in the absence and presence of 0.3, 1, and 3 µM of SEA0400. The initial uptake rates were identical with and without SEA0400, indicating that SEA0400 fails to modify the reuptake of Ca^{2+} from the intracellular space to the lumen of the SR.

IV/5. Effect of SEA0400 on the gating of single RyR2 channels

Effect of SEA0400 on single RyR2 channels was also studied using Müller type bilayer, where the solubilized RyR2 was incorporated into the lipid membrane, and single channel current was measured under voltage clamp conditions. The channels were almost fully open in the presence of 50 µM Ca^{2+} on the *cis* side: their open probability (P_o) was 0.978±0.047 (n=9). Reducing cis Ca^{2+} to 472 nM channels exhibited a P_o of 0.043±0.008 (n=9). After addition of 3 µM SEA0400 the open probability failed to change significantly (P_o = 0.032±0.005, n=6). It is also evident, that the conductance of RyR2 remained unaltered in the presence of SEA0400, since amplitudes of single channel currents were the same. Determining the mean open time of the channel revealed that two components are necessary to fit the open time histograms with a reasonable correlation coefficient. No significant differences were observed in the faster or slower time constants estimated before and after the application of 3 µM SEA0400. In summary, SEA0400 failed to modify any of the characteristic parameters of RyR2 up to the concentration of 3 µM.

IV/6. Effect of SEA0400 on Ca^{2+} sensitivity of contractile proteins in chemically skinned myocytes

As described in the Methods section, first the force-pCa relationship was determined by exposing the preparation to stepwise increases of external Ca^{2+} in order to determine the half-activation Ca^{2+} concentration, defined as pCa_{50}. The value estimated for pCa_{50} in these preparations was 6.4. The maximal isometric Ca^{2+}-activated force was 27.9±9.8 kN/m^2 and the passive force was 3.6±1.7 kN/m^2 in the 4 permeabilized cardiomycytes, each obtained from a different heart. Force

development was measured in the absence and presence of 1 µM SEA0400 at the single Ca^{2+} concentration of pCa=6.4. Therefore, any hypothetical SEA0400-dependent alteration in Ca^{2+}-dependent force production was not limited by Ca^{2+} saturation of the contractile machinery. SEA0400 was dissolved in concentrated DMSO, therefore, control measurements in the presence of DMSO were also included. The Ca^{2+}-activated force did not differ in the presence or absence of SEA0400 (0.55±0.03 and 0.55±0.04% of P_{max}, respectively). Moreover, force was also about the same when only DMSO was administered (0.57±0.03% of P_{max}) at pCa=6.4.

IV/7. Effects of SEA0400 on NCX

NCX current was recorded as a Ni^{2+}-sensitive current using the descending limb of a ramp pulse changing slowly from +60 to –100 mV during 1.6 sec. Outward and inward NCX current was determined at +40 and –80 mV, respectively. 1 µM SEA0400 suppressed both inward and outward NCX current, however, suppression of the outward current was more pronounced than the inward current block. The effect of SEA0400 on NCX current was studied at various levels of $[Ca^{2+}]_i$ (ranging from 55 nM to 1 µM) set by changing the $CaCl_2$ and EGTA content of the pipette solution. The blocking effect of SEA0400 on NCX was reduced by 50% with increasing $[Ca^{2+}]_i$ from 55 nM to 1 µM. It must be noted, however, that 1 µM SEA0400 was able to block 28±7 % of the inward NCX current even in the presence of 1 µM $[Ca^{2+}]_i$.

In theory, the missing effect of SEA0400 on myocyte shortening might be due to some direct effect of SEA0400 on SR Ca^{2+} handling, or by altering the Ca^{2+}-reponsiveness of the myofilaments. In the followings, therefore, these possibilities are examined.

IV/8. Effects of SEA0400 on $I_{Ca,L}$

Peak $I_{Ca,L}$ was measured at a rate of 0.2 Hz using depolarizing voltage pulses of 400 ms duration clamped from the holding potential of -40 mV to the test potential of +10 mV. K^+ currents were blocked by the externally applied 4-aminopyridine and internally applied TEACl. Stability of $I_{Ca,L}$ was monitored at least for 5 min before cumulative application of SEA0400 or KB-R7943 (from 0.3 to 30 µM, each concentration for 3 or 5 min, respectively). Both drugs caused

concentration-dependent suppression of peak $I_{Ca,L}$. Fitting results to the Hill equation yielded EC_{50} values of 3.6±0.14 and 3.2±0.36 µM and Hill coefficients of 1.1±0.01 and 0.9±0.1, respectively. Since both SEA0400 and KB-R7943 displayed approximately 75% of their full blocking effects at a concentration of 10 µM, this concentration was applied in the further studies.

Current-voltage relations for $I_{Ca,L}$ were obtained by applying a series of test pulses increasing up to +60 mV in 5 mV steps in Tyrode solution and in the presence of 10 µM SEA0400 and KB-R7943. Peak values of $I_{Ca,L}$ were plotted against their respective test potentials. No shift in the current-voltage relationship was observed after application of SEA0400 and KB-R7943. Ca^{2+} conductance (G_{Ca}) was calculated at each membrane potential by dividing the peak current by its driving force (the difference between the applied test potential and the reversal potential for $I_{Ca,L}$, estimated to be +55 mV). Ca^{2+} conductance was significantly reduced by both compounds at each membrane potential studied, however, when G_{Ca} values were normalized to the respective G_{Ca} obtained at +30 mV, the G_{Ca}-V_m relationships became directly comparable. The midpoint potential was –3.7±0.3 mV in Tyrode solution with the corresponding slope factor of 5.9±0.3 mV (n=13). Similar results were obtained with 10 µM KB-R7943 (midpoint potential of -3.9±0.3 mV and slope factor of 6.6±0.3 mV, N.S., n=7). SEA0400 caused a small, but statistically significant leftwards shift on the activation curve: the midpoint potential was shifted to -7.9±0.4 mV ($P<0.05$) without changing the slope factor (6.7±0.4 mV, N.S., n=6). These results indicate that voltage dependence of activation of $I_{Ca,L}$ is little affected by high concentrations of SEA0400 or KB-R7943.

In contrast to activation, the voltage dependence of inactivation was characteristically altered by 10 µM SEA0400 and KB-R7943. In order to study the voltage-dependence of steady-state inactivation of $I_{Ca,L}$, test depolarizations to +10 mV were preceded by a set of prepulses clamped to various voltages between -50 and +30 mV for 500 ms. Peak currents measured after these prepulses were normalized to the peak current measured after the –50 mV prepulse and plotted against the respective prepulse potential. The data were fitted to the two-state Boltzmann function. In Tyrode solution the midpoint potential of the steady-state inactivation curve was –15.3±0.6 mV with the corresponding slope factor of 3.9±0.2 mV (n=15). Superfusion of the cells with 10 µM SEA0400 shifted the midpoint potential by almost 15 mV to the left (to –29.5±1.3 mV, $P<0.001$, n=7), however, no

significant difference was obtained in the slope factor (4.2±0.3 mV). Similarly directed - although less pronounced - shift was observed with 10 µM KB-R7943: the midpoint potential was -19.7±1.1 mV ($P<0.01$, n=8) while the slope factor was 4.4±0.3 mV.

In addition to changes in voltage dependence of inactivation, the time constant of current decay was also modified by SEA0400 and KB-R7943. The time constant of inactivation was 18.7±0.8 ms in Tyrode solution at +10 mV (n=14), whereas time constants of 10±1.1 ms and 13.4±1 ms were obtained in the presence of 10 µM SEA0400 ($P<0.001$, n=7) and KB-R7943 ($P<0.001$, n=7), respectively.

Time course of recovery from inactivation was determined using twin-pulse protocol. Both pulses were 200 ms in duration, the interpulse potential was –40 mV, and interpulse interval was gradually increased up to 4 s. The time constant for recovery was estimated by fitting the data to a single exponential function, yielding recovery time constant of 316±15 ms in Tyrode solution (n=12). This value was significantly increased by 10 µM SEA0400 (to 1102±230 ms, $P<0.01$, n=5) and KB-R7943 (to 571±48 ms, $P<0.001$, n=7), respectively. These results indicate that inactivation of $I_{Ca,L}$ was accelerated, while the opposite transition (i.e. recovery from inactivation) was retarded in the presence of SEA0400 and KB-R7943.

Suppression of $I_{Ca,L}$ by KB-R7943 developed fully within 2 min, however, it was only partially reversible during the washout period. SEA0400 required longer time for development of its action, but similarly to KB-R7943, the reversibility was poor. For comparison, the effect of 10 µM fluoxetine - a drug shown to block $I_{Ca,L}$ in a comparable extent - was highly reversible under our experimental conditions.

V. DISCUSSION

In the course of our experiments it was our aim to investigate the effect of SEA0400 on the surface membrane ioncurrent, on the intracellular Ca^{2+} transient and on the features of contractile answer in order to define the probable mechanism of the therapeutic effects of SEA0400, and in order to decide to what extent can we judge the compound to be a selective NCX-blocker. Our short answer to the question is the following: with submicromol concentrations the SEA0400 is such a relatively selective NCX-blocker molecule which, according to its special qualities (a Ca^{2+} dependent dominantly reverse mode blocking), does not lead to the overloading of the cells with Ca^{2+}. With higher concentration the increasing NCX-blocking is well compensate by the also increasing blocking of the $I_{Ca,L}$ which defends the cell from being overloaded by Ca^{2+}. Thus we can effectively block NCX without increasing the Ca^{2+} loading of the cells.

V/1. SEA0400 blocks the Na^+/ Ca^{2+} exchanger selectively

In the course of our experiments we examined the effect of the NCX-blocking molecule on the transmembrane currents of myocytes and on Ca^{2+} handling in canine and guinea pig cardiac preparates. Several teams have already investigated the NCX blocking effect of SEA0400 but the problem of selectivity has not been resolved yet. One of the aims of our experiments was the examinations of selectivity on such preparates as canine ventricular myocytes which is very similar to human cardiac cells as far as Ca^{2+} handling is concerned.

Our results indicate that SEA0400 fails to alter the conductance or gating kinetics of the RyR2 Ca^{2+} release channel, has no effect on the rate of sarcoplasmic Ca^{2+} release and reuptake up to a concentration of 3 µM, or Ca^{2+} sensitivity of the myofilaments. These results - congruently with the unchanged $[Ca^{2+}]_i$ transients - indicate that SEA0400 exerts no direct action on sarcoplasmic Ca^{2+} handling in canine ventricular cells. Therefore, 0.3 µM SEA0400, which had negligible effects on transmembrane ion currents, can be considered a selective inhibitor of NCX, and as such, is a suitable tool to study the effects of partial NCX inhibition in cardiac myocytes. We can estabilsh that with low submicromlic concetrations SEA0400 exerts only a minimal blocking effect on the $I_{Ca,L}$ current, while it significantly blocks

the NCX current. With micromolic or higher concentrations we have to take into consideration the blocking effect of the molecule on the $I_{Ca,L}$ current, which can be definitly usefull with respect to its therapeutic effects.

It is worthwhile to compare SEA0400 and KB-R7943 compounds in this respect. On ventricular cells isolated from guiena pigs Tanaka et al. found that deducting the 10 % blocking of the $I_{Ca,L}$ current 1 µM SEA0400 did not modify one ioncurrent with the exeption of NCX current significantly, while with KB-R7943 10 µM concentration it blocked the various currents significantly (60-95%). At the same time the EC_{50} value of the blocking effect on NCX was lower in the case of SEA0400, than in the case of KB-R7943. Our own data extracted from canine NCX current and L-type calcium current are the same, with the difference that SEA0400 on guinea pigs blocked inward and outward currents with the same intensity, while we find dominant reverse mode blocking in our experiments on canine myocytes. As far as the interpretation of selectivity results is concerned in possession of data extruded from canine myocytes we think we had better estabilsh selectivity more carefully than Tanaka's team, who found SEA0400 to be a selective drug in 1 µM concentration. The 20% decrease of $I_{Ca,L}$ amplitude which we find in the presence of 1 µM SEA0400 leads to such an extent of Ca^{2+} influx that we can not forget about it during the analysis of the effect mechanism of the compound. We very high concentration (10 µM) it can block more than one ion channels and as a result the danger of proarrhythmias is increased.

V/2 The effect mechanism of SEA0400

SEA0400 did not modify the amplitude of left ventricular pressure and that of Ca^{2+} transient in the Langendorff perfused guinea pig heart and it had no effect on the intracellular Ca^{2+} transient of myocytes from the canine left ventricule, neither on cell shortening. From the examined parameters SEA0400 increased the time of decay of the descending limb significantly, which the only but very important evidence of the effect of the molecule. The time of decay of the relaxation of Ca^{2+} transient increased as a consequence of NCX-blocking, because transport system eliminating Ca^{2+} functioning beside NCX could not compensate the absence of NCX significantly.

On the basis of our examinations carried out on canine and guinea pig heart we can estabilsh that NCX-blocking inducated by SEA0400 did not lead to

intracellular. Ca^{2+} accumulation, because neither the amplitude of Ca^{2+} transient nor the left ventricular pressure and the quantity of cell shortening did not change in the presence of SEA0400 significantly. This is quite surprising because scientific literature finds NCX to be the deciding factor of Ca^{2+} elimination from the cell. The following mechanism are responsible for the fail of expectable positive inotrop effect in the case of canine and guinea pig hearts: 1. SEA0400 blocked NCX current functioning in reverse mode with greater affinity than the forward one, so it blocked the influx of Ca^{2+} with better effect than the efflux of Ca^{2+} via NCX. 2. The blocking effect of SEA0400 on NCX greatly depends on the free Ca^{2+} concentration of the cell plasm. With the increasing Ca^{2+} loading of the cell the NCX blocking decreases which makes possible the necessary Ca^{2+} elimination while at the same time the blocking effect on the Ca^{2+} channel remains. 3. With local blocking becoming more and more dominant via the increase of the SEA0400 concentration compensates the consequences of decreased Ca^{2+} elimination. Thus the quantity of influx of Ca^{2+} entering intracellular space decreases. Because of these effect of SEA0400 there is no intracellular Ca^{2+} accumulation in canine and guinea pig ventricular cardiac myocytes despite the blocking of NCX.

This is apparently in sharp contrast with the results obtained in rat and murine ventricular myocardium, where SEA0400 increased both contractility and $[Ca^{2+}]_i$ transients. This paradoxical situation can be resolved by considering the differences in the conditions that determine the operation of NCX, i.e. the actual values of transmembrane potential and cytosolic free sodium and calcium ion concentrations. Action potential duration is much shorter (50 ms *versus* 250 ms) and intracellular sodium concentration is much higher (15 mM *versus* 8 mM) in rats and mice than in other mammalian species including guinea pig. Therefore, NCX operates dominantly in forward mode during systole in rats and mice, while the longer action potential duration and lower intracellular sodium concentration allows NCX to operate in the reverse mode for a longer period of time during early systole in guinea pigs. As a consequence, symmetrical NCX inhibition (i.e. equal fractional blockade of the forward and reverse mode operation) is expected to cause a greater increase in $[Ca^{2+}]_i$ in rats or mice than in guinea pigs. On the other hand, since SEA0400 was shown to inhibit the reverse mode operation of NCX more effectively than its forward mode activity, SEA0400 is likely to suppress calcium influx *via* NCX more effectively in guinea pigs than in rats and mice. This favours calcium accumulation in

SEA0400-treated rat and murine hearts in contrast to guinea pig myocardium. Similarly to the present observations, SEA0400 had no effect on contractility in anaesthetized dogs. These results together suggest that NCX inhibition may increase $[Ca^{2+}]_i$ and contractility under physiological conditions only in species having short action potential duration, like mice or rats, but not in other mammals likely including humans.

V/3. The therapeutic possibilities of the application of SEA0400

The two most important biochemical changes after myocardiac ischaemia are acidosis developing as a result of the anaerob function and ATP depletion developing from decreased ATP production. Another consequence of ATP depletion is the decreased functioning of Na^+/K^+ pump, which leads to the accumulation of intracellular Na^+ which aggravated by Na^+ influx developing through NCX. The increased intracellular Na^+ concentration leads to Ca^{2+} elimination and the increased influx of Ca^{2+} via NCX. In cardiomyocytes surviving ischaemia the increased intracellular Ca^{2+} concentration can lead to cummulated arrhythmia during early reperfusion. The development of Ca^{2+}-accumulation and the generation of arrhythmias as a result can be blocked by the selective inhibition of NCX. By blocking NCX we can moderate both early and delay type of afterdepolarization. As a result of forward mode blocking the inward current will be smaller, which induces afterdepolarizational episodes, while reverse mode blocking decreases the quantity of Ca^{2+} entering the cell.

The blocking effect of SEA0400 induced via the NCX channel is increased by intracellular Na^+ and is decreased via intracellular Ca^{2+}. This explaines why SEA0400 protects the heart from Ca^{2+} overloading induced by ischaemia/ reperfusion. With ischaemia/reperfusion the NCX functions mainly in reverse mode because Na^+ concentration is high in myocytes. On the one hand SEA0400 effectivly blocks NCX functioning dominantly in reverse mode, on the other hand it blocks $I_{Ca,L}$ and accordingly the increased Ca^{2+} loading of myocytes fails as well as development of reperfusional arrhythmias.

The mutual blocking of the NCX current and the $I_{Ca,L}$ decreases the quantity of Ca^{2+} influx in myocytes. Due to this Ca^{2+} steady-state the influx of Ca^{2+} into myocytes is decreased so the quantity of the application of ATP is smaller in the case of smaller quantity Ca^{2+}. This effect of SEA0400 can be especially positive after

episodes of ischaemia and reperfusion, when ATP depletion is induced in myocytes. Summarily we can say that SEA0400 is a promising NCX blocking drug, which can play a major role in the curing of pathologic states with ischaemia/ reperfusion.

VI. SUMMARY

The Na^+/Ca^{2+} exchanger (NCX) plays a central role in Ca^{2+} handling of myocytes, it is the most important cellular mechanism in Ca^{2+} elimination. Among pathological circumstances it plays a role in the development of life threatening arrhythmias. With the selective blocking of NCX we can prevent the development of these arrhythmias. Many NCX blockers have been described in the literature, but all of them have proved to be applicable only to a limited extent for therapeutic application because of the absence of effective selectivity. According to studies the new NCX blocking molecule with a code number SEA0400, that has been recently syntetised, is held to be selective and therapeutically promising.

In the course of our experiments it was our aim to investigate the effect of SEA0400 on the surface membrane ionic currents, on the intracellular Ca^{2+} transient and on the features of the contractile answer in order to define the probable mechanism of the therapeutic effects of SEA0400, and in order to decide to what extent can we judge the compound to be a selective NCX-blocker. For our examinations we chose such preparates (cardiac myocytes isolated from canine and throbing guinea pig heart), which are very similar to human myocytes. We carried out our examinations with electrophysiological (patch-clamp) and optical (fluorimetric) methods. We established the following results:

1. The SEA0400 did not modify the amplitude of Ca^{2+} transient neither did it modify the left ventricular pressure or the cell shortening in myocytes from Langendorff-perfused guinea pig heart and canine left ventricule.
2. The SEA0400 failed to effect the Ca^{2+} release from HSR vesicules, the Ca^{2+} uptake from LSR vesicules, the gating features RyR2 receptors and Ca^{2+} sensibility of the concractile proteins of myocytes.
3. The SEA0400 blocked both the inward and the outward NCX current in a concentration-dependent manner on myocytes from canine left ventricule. The

SEA0400 induced a stronger blocking effect on the outward than on the inward NCX current, the increased intracellular Ca^{2+} concentration decreased its effect.

4. The SEA0400 blocked the L-type Ca^{2+} current in a concentration-dependent manner in ten times bigger quantity of concentration than the NCX current.

On the basis of our results we think that applied in submicromolic concentration SEA0400 is such a relatively selective NCX-blocking molecule, which does not lead to the overloading of the cells with Ca^{2+} due to its special features (Ca^{2+} dependent dominantly reverse mode blocking). With higher concentration the increased NCX blocking is well compensated by the also increased blocking of $I_{Ca,L}$, which protects the cell from being overloaded by Ca^{2+}. Thus it is possible to block NCX effectively without increasing the Ca^{2+} loading of the cells. Due to the above mentioned features of SEA0400 it can play a major role in the therapy of pathological states of ischaemia/reperfusion.

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