Investigation of the endogenous contributing factors of proarrhythmia and myocardial contractility

PhD thesis

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THE THESIS IS BASED ON THE FOLLOWING PAPERS

I. Importance of extracardiac α_1-adrenoceptor stimulation in assisting dofetilide to induce torsade de pointes in rabbit hearts

AS Farkas, K Acsai, N Nagy, A Tóth, L Dézsi, S Orosz, T Forster, M Csanády, JG Papp, A Varró, A Farkas

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II. Na^+/Ca^{2+} exchanger inhibition exerts a positive inotropic effect in the rat heart, but fails to influence the contractility of the rabbit heart

AS Farkas, K Acsai, N Nagy, A Tóth, F Fülöp, G Seprényi, P Birinyi, PP Nánási, T Forster, M Csanády, JG Papp, A Varró, A Farkas

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III. A Na^+/Ca^{2+} exchanger gátlás eltérő hatása az izolált patkány és nyúl szív kontraktilitására, Different effect of the inhibition of the Na^+/Ca^{2+} exchanger on the contractility in isolated rat and rabbit hearts

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IV. The role of The Na^+/Ca^{2+} exchanger, I_{Na} and I_{CaL} in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts

AS Farkas, P Makra, N Csík, S Orosz, MJ Shattock, F Fülöp, T Forster, M Csanády, JG. Papp, A Varró, A Farkas

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V. Endogenous factors and predictive parameters of dofetilide-induced torsades de pointes in anaesthetized, α_1-adrenoceptor-stimulated rabbits

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ACRONYMS AND ABBREVIATIONS

APD	action potential duration
AV	atrioventricular
BRS	baroreflex sensitivity
BG	bigeminy
EAD	early afterdepolarization
DAD	delayed afterdepolarization
DMSO	dimethyl sulfoxide
ECG	electrocardiogram
HF	heart failure
I_{CaL}	inward L-type calcium current
I_K	delayed rectifier potassium current
I_{Kr}	the rapid component of the delayed rectifier potassium current
I_{K1}	inward rectifier potassium current
I_{Na}	inward sodium current
i.v.	intravenous(ly)
LQTS	long QT syndrome
M cell	midmyocardial cell
Na_i	intracellular Na^+ level
NCX	Na^+/Ca^{2+} exchanger
QTc	heart rate corrected QT interval
$E_{Na/Ca}$	reversal potential of the NCX
TdP	torsades de pointes
TDR	transmural dispersion of repolarization
E_m	transmembrane potential
TWLI	T-wave lability index
VF	ventricular fibrillation
VPB	ventricular premature beat
VT	ventricular tachycardia
vs	versus

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1. INTRODUCTION

1.1. Background of the study

The long QT syndrome (LQTS) is caused either by inherited 'channelopathies' or by acquired factors, *e.g.* cardiac or non-cardiac drugs that hinder the process of repolarization of the myocytes. The most dangerous consequence of the disturbed repolarization is the occurrence of torsades de pointes polymorphic ventricular tachycardia (TdP). This life-threatening arrhythmia may cause palpitations, syncope, seizure-like activity and can be converted to ventricular fibrillation leading to death. Drug-induced TdP has been recognized for decades and the avoiding of proarrhythmia, e.g. TdP, has become a key element of contemporary new drug development. Indeed, QT prolongation and TdP have been the single most common cause of withdrawal of marketed drugs in the past decade [1]. Thus, the proarrhythmic liability of any drug under development must be assessed so as to avoid the occurrence of these drug-induced life-threatening arrhythmias during pharmacotherapy [2]. In the drug development and in the clinical practice there is a great demand to find different factors and parameters having a great predictive value for the occurrence of TdP. Animal models e.g. the Langendorff perfused rabbit heart model and α_1-adrenoceptor-sensitized anaesthetized rabbit models are available and widely spread to assess proarrhythmic effects of drugs [2].

A number of hypotheses have been proposed as concerns the generation of TdP, but the exact mechanism of this arrhythmia is still not clear. In the most widely accepted theory, the intrinsic transmural dispersion of repolarization (TDR) is amplified by any effect that reduces net repolarizing currents (e.g. drugs such as dofetilide, certain ion channel mutations, or remodelling of the ventricular wall) by preferential prolongation of the repolarization of the midmyocardial cells (M cells), which sets the stage for circus movement reentrant arrhythmias [3]. Furthermore, the reduction in net repolarizing currents also predisposes to the development of early afterdepolarization-induced (EAD) ventricular extrasystoles, which can provide the initiating beat that triggers reentrant tachyarrhythmias [3]. Thus, the mechanism of TdP initiation involves the EAD-induced ectopic beat, while the mechanism of the arrhythmia maintenance involves the reentry.

The cardiac Na^+/Ca^{2+} exchanger (NCX) might be a potential candidate of the formations of EADs, delayed afterdepolarizations (DADs) and reentries. The NCX is considered to play an important role in Ca^{2+} handling of cardiac myocytes. In forward mode, the NCX extrudes Ca^{2+} from the cell and brings Na^+ into the cytosol with a ratio of 1:3,

respectively; in reverse mode it operates in the opposite direction [4]. This exchange of 3 positive charges for 2 positive charges makes the exchanger electrogenic and this may cause EADs [5]. Moreover, NCX may have role in the development of transmural dispersion of repolarization [6]. Altogether, NCX might have a role in the development of TdP.

In addition, the operation of NCX may influence the heart muscle contractility. In heart failure (HF) the heart is unable to provide the essential cardiac output, since the contraction force of the heart is depressed. HF is an increasing health problem, affecting >2 million people in the U.S. alone [4]. There are two fundamental ways to increase the cardiac muscle contractility, cardiac inotropy: 1. increasing the intracellular Ca^{2+} level, so that more Ca^{2+} is supplied to the myofilaments and 2. enhancing Ca^{2+} sensitivity of myofilaments [4]. The inhibition of NCX by an endogenous inhibitor (NCX_{IF}) may result in transient increase in the cytosolic Ca^{2+}, enhancement of the Ca_i transient and cell-shortening [7], which can support the intracellular Ca^{2+} level elevating role of NCX rather than Ca^{2+} sensitivity effects on myofilaments. The intracellular Ca^{2+} level depends on the amount of Ca^{2+} entering the myocyte and the amount of Ca^{2+} removed from the myocyte. The Ca^{2+} enters the cell mainly via the I_{CaL} and only a smaller part of the entering Ca^{2+} is carried by reverse mode of NCX. Four mechanisms compete for cytoplasmic Ca^{2+} in Ca^{2+} removal: Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA), NCX, sarcolemmal CaATPase and mitochondrial Ca^{2+} uniporter. The major pathway for Ca^{2+} extrusion is the forward mode of NCX [4]. Thus, inhibition of the NCX may influence the cardiac muscle contractility. The examination of the physiological and the pathological operation of NCX were very difficult until a specific and potent NCX inhibitor, such as SEA0400 has been developed recently. Since the forward mode of NCX is considered as the main Ca^{2+} removal mechanism from the cytoplasm to the extracellular space, its selective inhibition should increase the contractile force of cardiac muscle.

1.2. Importance of extracardiac α_1-adrenoceptor stimulation and the constant left ventricular stretch in the development of torsades de pointes

A commonly used animal model for the *in vivo* screening of drug-induced proarrhythmia is the α_1-adrenoceptor-stimulated anaesthetized rabbit model of the acquired LQTS developed by Carlsson et al. [8]. The ability of a test agent to evoke TdP is evaluated during co-administration of a 'priming' substance, the selective α_1-adrenoceptor agonist methoxamine [8] or phenylephrine [9, 10]. The role of the α_1-adrenoceptor-stimulation, which makes the rabbits susceptible for the development of TdP in the presence of a repolarization

prolonging drug, is not completely understood. Although Carlsson et al. hypothesized that direct stimulation of the cardiac α_1-adrenoceptors is the facilitating factor in their model [8], this hypothesis has yet not been proven. On the other hand, it may also be hypothesized that reflex bradycardia and/or an increased ventricular stretch, which develop as a consequence of α_1-adrenoceptor-mediated peripheral vasoconstriction and increased resistance, are the critical sensitizing factors. However, this latter hypothesis has not yet been proven either.

Acute mechanical stretch induces complex changes in the heart muscle. An acute stretch during diastole may induce membrane depolarizations resembling DADs, whereas an acute stretch during systole usually shortens the action potential and the refractory period of the myocytes in a heterogeneous manner; however, action potential prolongation and the induction of EADs have also been reported [11]. Hence, an acute stretch can be arrhythmogenic by i) inducing triggered arrhythmias, e.g. extrasystoles, when stretch-induced EADs and DADs reach the threshold, or ii) facilitating reentrant arrhythmias by the heterogeneous shortening of the action potential and refractory period of the cardiomyocytes.

Despite intensive examinations of the arrhythmia genesis in the LQTS, so far no data have been published on the contribution of a ventricular stretch to the development of TdP. Similarly, despite the frequent use of the anaesthetized rabbit model of the acquired LQTS [8], hardly any experimental data have been published on the role of α_1-adrenoceptor stimulation in the model. Since a ventricular stretch, α_1-adrenoceptor stimulation and bradycardia can be separated from each other in isolated, Langendorff-perfused rabbit hearts, this model is suitable for examining the effects of these factors separately and in combination on the genesis of TdP.

1.3. Predictive parameters for torsades de pointes

ECG QT interval is a reflection of the aggregate of all cellular repolarization events in the heart [12]. There is a great demand to find different factors and parameters having great predictive value for the occurrence of drug-induced arrhythmias. Several potential parameters were suggested to predict the proarrhythmic liability better than the simple measurement of the rate corrected QT interval (QTc). The interval between the peak and the end of T wave (Tpeak-Tend) has been suggested to provide an index of ventricular transmural dispersion of repolarization (TDR), which may have prognostic value for proarrhythmic risk [13]. Thomsen et al. has proposed beat-to-variability of the QT intervals, which may determine the proarrhythmic outcome in a dog *in vivo* proarrhythmia model [14]. Beat-to-beat non-alternating T-wave lability, which was measured by the T wave lability index (TWLI),

occurred in patients with inherited LQTS during phenylephrine and dobutamine provocation and was associated with a history of prior cardiac events [15]. Hondeghem et al. introduced triangulation, reverse use dependence, instability and dispersion (TRIaD) as the primary causes of drug-related proarrhythmia [16]. The validation of these parameters has not yet fully performed. In the absence of confirmation of these parameters the QTc remains the most widely spread proarrhythmic predictor, though of its poor specificity and selectivity [16].

Interestingly, co-administration of dofetilide and the α_1-adrenoceptor agonist methoxamine failed to evoke TdP in isolated rabbit hearts [17]. This suggests that *in vivo* factors still unknown may have a crucial role in assisting the genesis of TdP. A recent study revealed that bilateral vagotomy prevented drug-induced TdP in the *in vivo* rabbit model of LQTS, which emphasized the role of the autonomic nervous system in TdP genesis [18]. However, the model has not been characterized sufficiently to date, and the mechanism of TdP genesis is not known. Also, there has not been any parameter found to date that can reliably predict drug-induced proarrhythmia in the model.

1.4. The role of the Na^+/Ca^{2+} exchanger, I_{Na} and I_{CaL} in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts

The electrogenic NCX may cause substantial depolarization in forward mode, leading to EAD- and DAD-induced triggered activity (extrasystoles) [5]. Selective inhibition of the NCX by SEA0400 reduced the amplitude of both dofetilide-induced EADs and the strophanthidin-induced DADs in isolated canine ventricular papillary and Purkinje fibres [19]. This implies that NCX activity may play a role in the generation of the trigger of drug-induced TdP.

The intrinsic transmural dispersion of repolarization is amplified by any effect that reduces net repolarizing currents (e.g. drugs such as dofetilide), by preferential prolongation of the repolarization of the M cells, which provide the substrate for circus movement re-entrant arrhythmias [3]. The NCX may contribute to the prolonged action potential of the canine M cells, which produces transmural electrical heterogeneity and re-entry circuits [6]. This implies that NCX activity may play a role in the generation of the maintenance mechanism of TdP.

The in vitro, isolated, AV ablated rabbit heart model is frequently used for testing the proarrhythmic liability of drugs [2]. Interestingly, the ability of pharmacological interventions to reduce the incidence of TdP induced by K^+ channel blocking drugs has not been examined in this model.

1.5. The effect of Na^+/Ca^{2+} exchanger inhibition on the cardiac muscle contractility in isolated rat and rabbit hearts

The direction of operation of the NCX depends mainly on the transmembrane gradients of Ca^{2+} and Na^+ and the prevailing transmembrane potential (E_m). In the early phase of the action potential the NCX works in reverse mode and brings Ca^{2+} into the cell, while during repolarization it removes Ca^{2+} from the cell in the forward mode. Thus, the duration of the action potential influences the operation of the NCX [20].

The duration and shape of the action potential may vary with the species, due to the uneven distribution of the various transmembrane K^+ channels. For instance, the duration of the action potential, and particularly that of the plateau phase, are much longer in rabbit myocytes as compared with those in rat myocytes, which suggests that the NCX operates differently in rabbit and rat myocytes.

The cardiac contractility and force development depend on the free intracellular Ca^{2+} level and the Ca^{2+} sensitivity of the myofilaments. On a beat-to-beat basis, in order to increase the intracellular Ca^{2+} level, the Ca^{2+} entry should be increased or the Ca^{2+} removal should be decreased. The NCX is regarded as the main Ca^{2+}-extruding mechanism in the myocardium and its inhibition may alter the intracellular Ca^{2+} level and the cardiac muscle contractility.

A change in the expression (e.g. an overexpression) of the NCX protein can also affect the cardiac muscle contractility. The overexpression of the NCX in genetically manipulated feline myocytes caused a decline in contractility [21]. The contraction and Ca^{2+} transient decreased in NCX overexpressing rat cardiomyocytes by adenoviral-mediated gene transfer [22]. A weakened cardiac muscle contractility has likewise been observed in myocardial hypertrophy, heart failure and even myocardial infarction, due to the increased expression and altered activity of the NCX [23]. However, no study has yet been reported in which the levels of NCX expression in different species under physiological conditions were compared. Furthermore, the relationship between the level of NCX expression and the NCX function has not been elucidated.

1.6. Aims of the study

The main aim of our investigations was to identify the endogenous contributing factors of drug-induced TdP. The NCX, which was thought to be one of the key factors of the genesis of TdP, was examined in order to determine its contribution to i) TdP development and ii) cardiac contractility.

The aim of '*in vitro* α_1-adrenoceptor stimulation and stretch study' was to investigate whether triggered arrhythmias induced by a constant high level of left ventricular stretch are able to initiate TdP in the presence of functional reentries caused by the repolarization-lengthening Class III. antiarrhythmic drug, blocker of the rapid component of the delayed rectifier potassium current (I_{Kr}) dofetilide in the presence or the absence of α_1-adrenoceptor stimulation in isolated Langendorff-perfused rabbit hearts.

The aims of the '*in vivo* dofetilide study' were to identify endogenous factors of drug-induced TdP and to examine the mechanism of TdP genesis in anaesthetized, α_1-adrenoceptor-stimulated rabbits. Our study investigated whether the occurrence of dofetilide-induced TdP is predicted by the basic haemodynamics (*i.e.* the blood pressure and the heart rate), the blood gases, the frequency of the preceding arrhythmias, the repolarization-related parameters or the activity of the autonomic nervous system in pentobarbital anaesthetized, α_1-adrenoceptor-stimulated rabbits.

The potential role of the NCX in the genesis of drug-induced TdP has been examined in the 'NCX arrhythmia study'. Accordingly, the effect of the inhibition of the NCX by a selective NCX inhibitor (SEA0400) was investigated on the occurrence of dofetilide-induced TdP in isolated, Langendorff-perfused, atrioventricular nodal (AV)-ablated rabbit hearts. Our results show that NCX inhibition with SEA0400 did not reduce the incidence of dofetilide-induced TdP. Since this result questions either the role of NCX in TdP or the validity of this model, we conducted a second series of experiments aimed at validating the model with drugs known to alleviate TdP in other experimental models and in man. Thus, the anti-arrhythmic effect of the inhibition of the L-type Ca^{2+} current (I_{CaL}) by verapamil and the inhibition of the Na^+ current (I_{Na}) by lidocaine was tested against dofetilide-induced TdP in isolated, Langendorff-perfused, AV-ablated rabbit hearts.

Our objective was in the 'NCX contractility study' to examine the effects of selective NCX inhibition by SEA0400 on the cardiac muscle contractility in isolated Langendorff-perfused hearts in the setting of the long action potential of the rabbit and in the short action potential of the rat. To clarify the inhibitory effect of SEA0400 on the reverse and the forward modes of the NCX in the rabbit and in the rat isolated myocardial cell, $I_{Na/Ca}$ was examined by a patch clamp technique. NCX protein densities of the two species were compared to clarify whether interspecies differences in the contractile function can be explained by differences in NCX expression.

2. MATERIALS AND METHODS

2.1 Animals

Female New Zealand White rabbits were used for the *in vivo* TdP experiments. *In vitro* TdP experiments were performed on hearts excised from female New Zealand White rabbits to assess the role of stretch and the inhibition of NCX on the genesis of TdP. The cardiac muscle contractility experiments were carried out on male New Zealand White rabbits and male Sprague-Dawley rats. The animals were handled in accordance with the European Community guidelines for the use of experimental animals, and the protocol was reviewed and approved by the Ethical Committee for the Protection of Animals in Research at the University of Szeged, Hungary.

2.2 Langendorff perfusion of the isolated hearts

The hearts were retrogradely perfused at a constant temperature of 37°C with the modified Krebs-Henseleit buffer solution containing (in mM) NaCl 118.5, $CaCl_2$ 2.0, glucose 11.1, $MgSO_4$ 0.5, NaH_2PO_4 1.2, $NaHCO_3$ 25 and KCl 3. In the NCX contractility study, Krebs-Henseleit buffer solution contained the same salts but the concentrations of $CaCl_2$, $MgSO_4$, and KCl were 2.0 mM, 1.0 mM and 4 mM, respectively.

2.3. Study designs of the in vitro studies

The choice of drug solution was made by reference to a randomization table in each in vitro study. Randomization was achieved by coding each group with a letter whose meaning was unknown to the operator. Blinded analysis was achieved by using stock solutions prepared by a second operator, who did not participate in the heart perfusion or data analysis. The protocol of studies involved a randomized design with a time-matched control group, and blinded experimentation, data collation and analysis. For the assessment of the vehicle control or each individual concentration of the drug, each heart was used only once.

2.4. Assessment of α_1-adrenoceptor stimulation and the constant left ventricular stretch in the development of torsades de pointes in isolated rabbit hearts

2.4.1. Experimental protocol

In the first set of experiments, to find the appropriate dofetilide concentration in terms of the proarrhythmic potential, three groups of hearts [dofetilide 50 nM (n=8), dofetilide

100 nM (n=9), and control (solvent of dofetilide, n=10)] were compared. In each heart, the drug perfusion was started after 15 min of initial perfusion with modified Krebs-Henseleit solution, and lasted for 40 min. TdP occurred only in the 100 nM dofetilide group and it did so with an incidence which offered scope for the examination of additional effects that can further increase the incidence of this arrhythmia. This concentration was therefore chosen and used in the remainder of the study.

In the second set of experiments, three groups of hearts (n=8 hearts in each group) were perfused with 100 nM dofetilide for 40 min after 15 min of initial perfusion with modified Krebs-Henseleit solution. In two of the three dofetilide-perfused groups of hearts, methoxamine at a concentration of 100 nM was added to the dofetilide-containing perfusion solution. In each heart, a non-elastic balloon was inserted into the left ventricle via the mitral valve and was connected to a pressure transducer. The intraventricular balloon was filled with water at a constant volume of 1.4 ml throughout the whole experiment in the group of hearts perfused only with dofetilide and the vehicle of methoxamine (‘dofetilide+stretch’ group) and in one of the groups of hearts perfused with dofetilide and methoxamine (‘dofetilide+methoxamine+stretch’ group).

Individual measurements of coronary flow, left ventricle pressure and ECG variables were made every 5 min and 1 min before and 1 min after the introduction of drug perfusion. At the end of each experiment, the atria were removed from the heart and the ventricles were weighed.

2.5. In vitro and in vivo arrhythmia diagnosis and ECG analysis

ECG intervals were measured at predetermined time points in all experiments of each study. After the completion of experiments, the data were replayed and the RR, PR, QRS and QT intervals were measured by manual positioning on screen markers in all study except the NCX TdP study, where PR and QRS intervals were not measured after the AV node ablation. The QT interval was defined as the time between the first deviation from the isoelectric line during the PR interval until the end of the TU wave. Where the T or U wave overlapped the following P wave or the QRS complex of the subsequent sinus beat, the extrapolation method was used to measure the length of the QT (or QU) interval [24].

From the ECG, the incidence and the time to onset of arrhythmias were obtained. Ventricular premature beats (VPB), bigeminies (BG), salvoes and ventricular fibrillation (VF) were defined according to the Lambeth Conventions [25]. Torsades de pointes was defined as a polymorphic ventricular tachycardia with runs of 4 or more ventricular premature beats,

where clear twisting of the QRS complexes around the isoelectric axis could be seen in at least one ECG lead. Runs of 4 or more ventricular premature beats without the torsade-like twisting QRS morphology were differentiated from TdP and were defined as ventricular tachycardia (VT). When continuous ventricular fibrillation lasted longer than 120 s, the experiment was terminated.

2.6. The assessment of predictive parameters of dofetilide-induced torsades de pointes in α_1-adrenoceptor stimulated pentobarbital anaesthetized rabbits

2.6.1. Anaesthesia and surgical preparation

30 rabbits were used for the experiments. The animals were anaesthetized intravenously with pentobarbital (53±2 mg/kg) via the marginal vein of the left ear. The rabbits were retrospectively divided into 2 groups according to the presence or the absence of TdP, i.e. the animals that experienced TdP formed the 'TdP+' group, and the animals that did not experience this arrhythmia formed the 'TdP-' group. The administered doses of pentobarbital did not differ significantly between the 'TdP+' and the 'TdP-' group (data not shown).

After anaesthesia induction, a catheter was introduced into the right carotid artery to measure blood pressure. Two other catheters were introduced into the right jugular vein and the marginal vein of the right ear for the infusion of drugs. After tracheal cannulation, artificial ventilation was started. Subcutaneous needle electrodes were inserted in all four limbs, and leads I, II, and III of the electrocardiogram (ECG) were recorded simultaneously.

2.6.2. Experimental protocol

After a 10-min baseline period, α_1-adrenoceptor agonist phenylephrine infusion was started at increasing rates (*i.e.* 3, 6, 9, 12, and 15 μg/kg/min for 3, 3, 3, 3, and 5 min, respectively). From the 27^{th} min, dofetilide (0.02 mg/kg/min iv., for 30 min) was administered simultaneously with the background phenylephrine infusion (at a rate of 15 μg/kg/min) until the end of the experiments.

2.6.3. Measurements of the repolarization parameters

All the values for the QT interval were corrected for the heart rate with the equation QTcL = QT-0.704(RR-250) developed for pentobarbital-anaesthetized rabbits [26]. The T_{peak}-T_{end} interval was measured according to [27] in the standard limb Lead II of the ECG in

the last min before the start of the phenylephrine infusion (baseline), in the last min before the start of the dofetilide infusion, and in the 4th min of the dofetilide infusion (further measurements were prevented by the frequent occurrence of dofetilide-induced arrhythmias). The T wave lability was quantified as a root-mean-square of the differences between corresponding signal values of subsequent beats. The magnitude of aperiodic T-wave lability was quantified by measuring a T-wave lability index (TWLI) with a computer program developed buy us following the descriptions of Nemec et al. [15].

2.6.4. Measurement of the beat-to-beat variability of the QT and RR intervals

The beat-to-beat variability of the QT intervals was determined from the manual measurement data on 40 consecutive QT intervals and the corresponding RR intervals in sinus rhythm ('sinus variability') at predetermined time points, *i.e.* in the last min of the drug-free state, in the last min of the phenylephrine infusion before the dofetilide administration, and in the 2nd-4th min of the dofetilide infusion, when arrhythmias were still infrequent.

The 'real' beat-to-beat variability of the QT and RR intervals was determined irrespectively from the presence or absence of sinus rhythm from manual measurement data on 40 consecutive QT intervals and the corresponding RR intervals at the above mentioned three time points and immediately before TdP occurred. That is, 40 consecutive ventricular cycles (RR intervals with the corresponding QT intervals) were measured even when any kind of arrhythmia was present on the ECG at the predetermined time point of the measurement.

A computer program was developed in a .NET environment to obtain the following parameters of the beat-to-beat variability of the QT and RR intervals: For a sequence of RR or QT interval durations, the 'root mean square' (RMS) was calculated according to the following definition: $\mathrm{RMS} = \sqrt{\frac{1}{N}\sum_{i=0}^{N-1} d_i^2}$, where d_i represents the sequence of RR or QT interval durations and N is the total number of intervals. The 'root mean square' (RMSSD) and the 'standard deviation' (SDSD) of the successive differences of the RR and QT intervals, the percentage of successive QT intervals that differ by more than 8 ms (PNN8), the 'QT variability index' (QTVI), the 'instability' of the RR and QT intervals, the 'short-term variability' (STV) and the 'long-term variability' (LTV) were calculated as described earlier [28]. We also calculated the 'total instability' (TI), the 'short-term instability' (STI) and the 'long-term instability' (LTI) of the consecutive RR and QT intervals as described by van der Linde et al. [29].

2.6.5. Analysis of the spontaneous baroreflex sequences and spectral analysis of the variation in the systolic arterial blood pressure and the duration of the RR intervals

The baroreflex sensitivity was calculated as described earlier [28]. Baroreflex analysis was performed in the last 5 min of the drug-free period and in the last 1 min of each rate of phenylephrine infusion before the dofetilide administration. Spectral analysis was carried out as described earlier [28]. Spectral analysis was performed with the arterial blood pressure and the ECG signal of the animals recorded in the last 5 min of the drug-free period and the 3 min of the 9 μg/kg/min phenylephrine infusion rate.

2.7. Examination of the role of the Na^+/Ca^{2+} exchanger, I_{Na} and I_{CaL} in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts

2.7.1. Experimental protocol

In the first set of experiments, four groups (n=8 hearts in each group) [dofetilide 100 nM (DOF1), SEA0400 1.0 μM + dofetilide 100 nM (SEA+DOF), and control groups: distilled water (H_2O) and dimethyl sulfoxide, solvent of dofetilide and SEA0400, (DMSO)] were compared (Table 1). H_2O control group was involved as DMSO may affect the repolarization [30, 31]. In the 2nd minute, the AV node was ablated using forceps two minutes after mounting the heart. After AV ablation the hearts were allowed to beat in their own spontaneous rhythm.

The administration of the NCX inhibitor SEA0400 or the solvent of the SEA0400 was started at the beginning of a 20-min-long 'pretreatment' period, after 15 min of initial perfusion with modified Krebs-Henseleit solution. Dofetilide was added to the perfusion solution at the beginning of a 30-min-long 'treatment' period (Table 1). To block the NCX, SEA0400 was chosen because it is considered the only highly selective and potent NCX inhibitor. 1 μM concentration of SEA0400 was chosen in order to get maximal NCX inhibition without significantly affecting any other ion current [32-35].

Since SEA0400 administration did not decrease the incidence of dofetilide-induced TdP ventricular tachycardia, a second set of experiments was designed to examine whether it is possible to reduce dofetilide-induced TdP with other drugs in the applied model. Lidocaine and verapamil were chosen as test drugs as they could successfully reduce the incidence of drug-induced TdP in other experimental models [36, 37]. The second set of experiments comprised three groups of hearts: i) 100 nM dofetilide (DOF2), ii) 30 μM lidocaine+100 nM

dofetilide (LID+DOF), iii) 750 nM verapamil+100 nM dofetilide (VER+DOF). The protocol is outlined in Table 1.

Group	n	Pretreatment (20 min)	Treatment (30 min)
1st set of experiments			
H_2O	8	H_2O	H_2O
DMSO	8	DMSO	DMSO
Dofetilide 100 nM	8	DMSO	DMSO+dofetilide
SEA0400 1.0 µM+Dofetilide 100 nM	8	DMSO+SEA0400	DMSO+SEA0400+dofetilide
2nd set of experiments			
Dofetilide 100 nM	8	DMSO	DMSO+dofetilide
Lidocaine 30 µM+Dofetilide 100 nM	8	DMSO+lidocaine	DMSO+lidocaine+dofetilide
Verapamil 750 nM+Dofetilide 100 nM	8	DMSO+verapamil	DMSO+verapamil+dofetilide

Table 1. The experimental protocol applied in isolated, Langendorff-perfused, AV-blocked rabbit hearts. The groups of hearts, the periods of the experiments and the applied drugs are summarized. In the baseline period of the experiments, all hearts were perfused with Krebs buffer; the perfusion solution was then switched to the test drug in the second period of the experiment (pretreatment); in the last period dofetilide was added to the perfusion (treatment). Group size is indicated by n.

2.7.2. Measurement of the beat-to-beat variability of the QT intervals and the RR intervals

Please, see chapter 2.6.4.

2.8. Analysis of the effect of Na^+/Ca^{2+} exchanger inhibition on the cardiac muscle contractility in isolated rat and rabbit hearts

2.8.1. Measurements of the cardiac contractile force

The contractile function of the left ventricle in isolated rabbit and rat hearts was measured by using a non-elastic balloon filled with water, connected to a pressure transducer. The balloon was inserted into the left ventricle via an incision in the left atrium and the mitral valve. The ventricular systolic, end-diastolic pressures and the developed pressure (i.e, the systolic pressure minus the diastolic pressure) were recorded.

2.8.2. SEA0400 concentrations and Langendorff contractility protocol

With regard to the literature and our own measurement of $I_{Na/Ca}$, SEA0400 concentrations of 0.1, 0.3 and 1.0 µM were chosen for the experiments, since 0.1 and 0.3 µM are around the measured IC_{50} and SEA0400 is considered selective for the NCX up to a concentration of 1.0 µM.

A control group (which received the solvent of SEA0400) and the three treated groups (which received the different concentrations of SEA0400: 0.1, 0.3, and 1.0 µM) were

compared as concerns both the rabbit heart and the rat heart. Each of the groups in the rabbit study contained 10 hearts, and each of the four groups in the rat study contained 12 hearts. Each heart was set up under Krebs perfusion and the balloon was inserted into the left ventricle. Then, 0.1-ml increments in the rabbit heart and 0.02-ml increments in the rat heart were added to the balloon volume in every minute to reach the maximum developed pressure (a Starling curve was constructed), the final diastolic pressure not being allowed to exceed 10 mm Hg. Then whole procedure was repeated in the presence of the test drug.

Individual measurements of coronary flow, left ventricle pressure and ECG variables were made 1 min before, 4 min after and every minute during the Starling curves. The first Starling curve was constructed to test the health of each heart and to provide drug-free baseline data. The two Starling curves (before and during drug perfusion) were used to evaluate the inotropic and lusitropic effects more fully. At the end of each experiment, the atria were removed from the heart and the ventricles were weighed. The protocol is outlined in Figure 1.

a

	Krebs buffer			**SEA0400**		
Exp. time (min)	1-10	11-25	26-30	**31-40**	**41-55**	**56-60**
Intervals (min)	**10**	**15**	**5**	**10**	**15**	**5**
Balloon V (ml)	0.5	Starling curve	0.5	0.5	Starling curve	0.5

b

	Krebs buffer			**SEA0400**		
Exp. time (min)	1-10	11-19	20-24	**25-34**	**35-43**	**44-48**
Intervals (min)	**10**	**9**	**5**	**10**	**9**	**5**
Balloon V (ml)	0.08	Starling curve	0.08	0.08	Starling curve	0.08

Figure 1. The protocol applied in isolated, Langendorff-perfused hearts to assess cardiac contractility. Protocol for rabbit hearts (a) and protocol for rat hearts (b). Exp. time: experimental time; Balloon V: volume of the left ventricular balloon; Starling curve: starting from the "zero volume" 0.1-ml increments for the rabbit heart and 0.02-ml increments for the rat heart were added to the balloon volume every minute.

2.8.3. Measurement of $I_{Na/Ca}$ in the rat and the rabbit myocytes

To examine the effects of SEA0400 on the NCX current, the whole-cell configuration of the patch clamp technique was applied [35]. $I_{Na/Ca}$ was recorded by using ramp pulses. 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. The outward and the inward $I_{Na/Ca}$ were determined during the descending limb of the ramp, at +40 and –80 mV, respectively.

2.8.4. Confocal laser scanning microscopy and quantification of fluorescence intensities

Confocal laser scanning microscopy was applied for the quantification of the NCX on the sarcolemmal surface of cardiac myocyte [38]. The fluorescent images of 25 randomly selected rat and 25 rabbit cardiac myocytes, in each case derived from 4 animals, were subjected to immunofluorescent profile analysis.

2.9. Drugs

$CaCl_2$, DMSO, methoxamine, phenylephrine, lidocaine and verapamil and drugs for the isolation of myocytes were purchased from Sigma-Aldrich Co. (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.). All other salts were purchased from Molar Chemical Ltd. (Budapest, Hungary). The dofetilide was a generous gift from Gedeon Richter Ltd. (Budapest, Hungary). Pentobarbital (sodium pentobarbital, Nembutal®) was purchased from Phylaxia-Sanofi (Budapest, Hungary); and heparin-sodium from Gedeon Richter Ltd. (Budapest, Hungary). The synthesis of SEA0400 was performed by Ferenc Fülöp (Dept. of Pharmaceutical Chemistry, University of Szeged, Szeged, Hungary) according to a method described in the literature [39].

2.10. Statistics

Continuous data were expressed as mean ± standard error of the mean (s.e.mean). All data from independent samples, with the exception of arrhythmia incidences, were compared with Kruskal-Wallis tests. Continuous data from the same sample were compared with Wilcoxon tests. Arrhythmia incidences were compared by using the Fisher's exact probability test. In NCX contractility study, continuous data from independent samples were subjected to repeated measures analysis of variance, while NCX protein density data from independent samples were compared through one-way analysis of variance. Differences were considered statistically significant when $p<0.05$.

3. RESULTS

3.1. *In vitro* α_1-adrenoceptor stimulation and stretch study

3.1.1. Arrhythmia incidences

In the first set of experiments, spontaneous TdP occurred only in the 100 nM dofetilide group and it did so with a relatively low incidence (Table 2), which offered scope for the examination of additional effects that can further increase the incidence of this arrhythmia. On the other hand, dofetilide at a concentration of 100 nM evoked ventricular conduction blocks in a majority of the hearts (Table 2).

In the second set of experiments, neither the sustained load-induced left ventricular stretch nor methoxamine nor the in combination increased the incidence of dofetilide-provoked TdP. However, the stretch was arrhythmogenic in terms of increasing the incidence of salvo and of VT different from TdP as compared with the control group, and VPB occurred in each heart only in those two groups in which the stretch was applied (Table 2).

		Incidence of arrhythmias (%)					
Group	**n**	**VPB**	**BG**	**Salvo**	**Block**	**VT**	**TdP**
Control	10	70	10	0	10	0	0
50 nM dofetilide	8	50	0	25	25	0	0
100 nM dofetilide	9	89	56	56	89*	44	44
100 nM dofetilide+stretch	8	100	63	88*	63	63*	0
100 nM dofetilide+methoxamine	8	75	25	38	75	25	25
100 nM dofetilide+methoxamine+stretch	8	100	38	75*	38	63*	0

Table 2. Incidence of arrhythmias evoked by dofetilide, stretch and methoxamine in isolated rabbit hearts. Values are percentage incidences of arrhythmias. VPB: ventricular premature beat; BG: bigeminy; Block: conduction block of any kind; VT: ventricular tachycardia different from torsades de pointes; TdP: torsades de pointes ventricular tachycardia. Group size is indicated by n. *$P<0.05$ as compared with the Control group.

3.1.2. ECG intervals

Dofetilide reduced the heart rate similarly in all groups of hearts as compared with the control (Figure 2a). On the other hand, dofetilide caused a concentration-dependent lengthening of the QT interval and neither the stretch nor methoxamine significantly affected the dofetilide-induced QT prolongation (Figure 2b). As there was no significant difference between the heart rates of the dofetilide-treated groups (Figure 2a), the QT values were not corrected for heart rate. Dofetilide also caused a concentration-dependent lengthening of the QRS interval (Figure 2c).

The mean baseline PQ interval ranged from 60±1 to 74±2 ms and remained stable throughout the experiments; there was no significant difference between the PQ values of the various groups at any time point of the experiments. Neither the stretch nor methoxamine influenced the length of the PQ interval (data not shown).

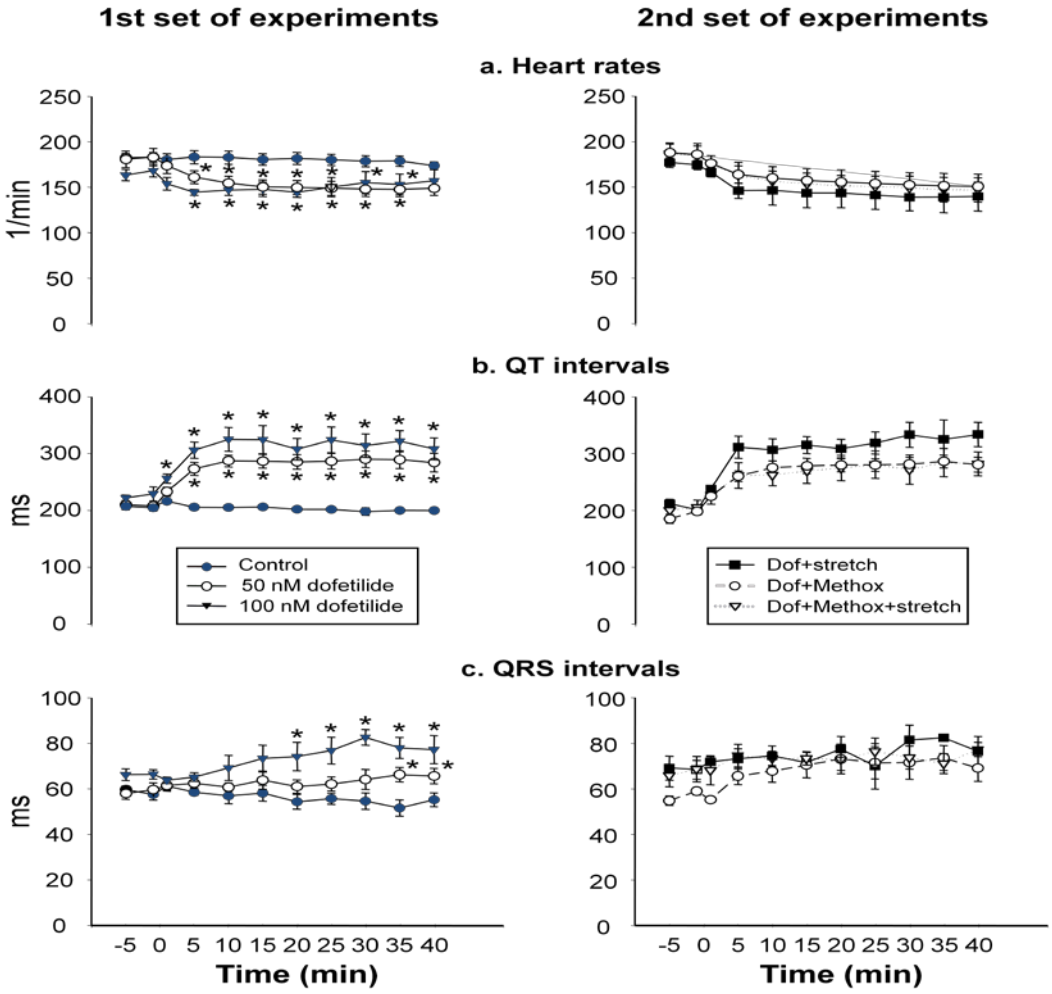

Figure 2. Heart rates (a), QT intervals (b) and QRS intervals (c) in isolated rabbit hearts. Drug perfusion started at ”0” min. Values are mean ± S.E.M. $^{*}P<0.05$ as compared with the Control group. For further details, see chapter *2.5.1.*

3.1.3. Left ventricular pressures

Neither the systolic nor the end-diastolic left ventricular pressure differed significantly between the ‘dofetilide+stretch’ group and the ‘dofetilide+methoxamine+stretch’ group. The left ventricular systolic pressure ranged from 139±4 to 157±11 mm Hg and from 141±7 to 153±5 mm Hg, respectively. The mean end-diastolic pressure did not exceed 9±2 mm Hg in any of the groups at any time point of the experiments.

3.2. Predictive parameters in the '*in vivo* dofetilide study'

3.2.1. Arrhythmia incidences and the onset time of TdP

TdP and ventricular fibrillation did not occur before the dofetilide infusion. The incidence of all kind of arrhythmias was higher, but not significantly, in the 'TdP+' group before dofetilide administration (Table 3). Dofetilide infusion markedly increased the incidence of arrhythmias in both groups. Interestingly, not only TdP, but other complex arrhythmias occurred more frequently in the 'TdP+' group than in the 'TdP-' group, i.e. also the incidence of non-TdP (VT) was significantly higher in the 'TdP+' group than in the 'TdP-' group during dofetilide infusion. TdP degenerated into sustained ventricular fibrillation leading to death in 3 animals (15%) in the 'TdP+' group (Table 3).

There was no statistical difference in the onset times of VPBs, salvos and VTs between the various groups (data not shown).

			Incidence of arrhythmias (%)						
		n	VPB	BG	Salvo	Block	VT	VF	TdP
Before dof.	**TdP-**	10	30	20	20	0	10	0	0
	TdP+	20	65	45	35	10	15	0	0
During dof.	**TdP-**	10	90	80	90	60	40	0	0
	TdP+	20	100	95	100	90	100*	15	100

Table 3. Incidence of arrhythmias in anaesthetized rabbits. Values are percentage incidences before and after dofetilide perfusion. Group size is indicated by n. *P<0.05 as compared with the TdP- group. For further details, see Table 2.

3.2.2. Blood pressure

The baseline systolic and diastolic arterial blood pressure (SAP and DAP, respectively) values did not differ between the two groups (Table 4). The stepwise elevation of the rate of infusion of phenylephrine increased the blood pressure before the dofetilide infusion. Arrhythmias occurred frequently in all animals approximately 4 min after the start of the dofetilide administration, which prevented measurement of the blood pressure in sinus rhythm thereafter. There was no significant difference in the blood pressure values between the two groups at any time point of the measurement.

3.2.3. Heart rate and ECG intervals

Phenylephrine reduced the heart rates and prolonged the QT intervals in both groups before the dofetilide infusion, but there were no differences in the heart rates and the QT intervals between the groups immediately before the start of the dofetilide infusions (Table 4).

Arrhythmias occurred frequently in all animals approximately 4 min after the start of the dofetilide administration, which prevented measurement of the sinus heart rate and the ECG intervals thereafter.

The dofetilide infusion prolonged the QT (Table 4) and the rate-corrected QT intervals (QTc) in both groups up until the time point at which these intervals could be measured (see above). There was no significant difference in the QT and the QTc intervals between the two groups. There was no qualitative difference in the effects of the drugs on the QT and the QTc intervals at any time point in either group of animals, thus QTc data is not shown.

There was no significant difference in the T_{peak}-T_{end} intervals between the two groups at any of the three time points of the measurement. However, within group analysis revealed that phenylephrine prolonged the T_{peak}-T_{end} interval significantly in the 'TdP+' group as compared with the baseline values (Table 4). A non-significant, slightly less elevation in the T_{peak}-T_{end} interval was found in the 'TdP-' group during phenyelphrine infusion. Interestingly, dofetilide infusion in the first 5 min did not prolong the T_{peak}-T_{end} interval significantly as compared with the value measured before the start of this infusion (Table 4).

T-wave lability index (TWLI) could be measured only until the end of phenylephrine infusion, since dofetilide evoked many arrhythmias, which did not allow assessing 100 subsequent T wave during normal sinus rate. Phenylephrine slightly increased the TWLI in the 'TdP+' group, but the change remained insignificant (Table 4).

	Baseline			**Phenylephrine**				**Phe+Dof**
exp. time	0 min	10 min	13 min	16 min	19 min	22 min	27 min	32 min
TdP-								
HR	275±10	275±10	270±10	255±12	253±13	242±15	236±12	222±23
QT	160±4	167±3	163±4	172±5	172±6	184±6	188±7	213±14
TpTe		52±5					58±5	68±7
TWLI		0.08±0.02					0.08±0.01	n.d.
DAP	85±5	94±4	99±5	105±4	110±5	114±4	115±3	126±7
SAP	110±4	117±3	122±5	129±5	137±5	139±5	143±5	151±6
TdP+								
HR	285±7	280±7	276±6	258±7	243±7	233±7	231±7	205±23
QT	168±4	166±4	166±4	172±4	177±5	184±6	189±6	199±15
TpTe		51±3					60±5	60±5
TWLI		0.08±0.01					0.12±0.02#	n.d.
DAP	90±3	93±2	102±3	106±3	109±3	112±4	115±2	111±6
SAP	115±3	117±3	126±3	131±4	135±3	139±4	146±4	140±6

Table 4. Heart rates (1/min), QT (ms), T_{peak}-T_{end} (ms) intervals, T-wave lability index and mean arterial blood pressures (mm Hg) in anaesthetized rabbits. n.d.: value not determined due to the frequent occurrence of arrhythmias. All values are means ± S.E.M. *P<0.05 vs. the value measured before the start of phenylephrine infusion at 10 min; $^{\#}$P<0.05 vs. the value measured before the start of dofetilide infusion at 27 min.

The mean baseline PQ interval were 50±1 ms in both groups and remained stable up until the time point at which this parameter could be measured (4 min of dofetilide infusion, see above); there was no significant difference in the PQ values between the two groups at any time point of the measurement. The mean baseline QRS interval was 47±1 ms and 49±3 ms in the 'TdP+' and the 'TdP-' group, respectively. The QRS interval remained stable in both groups up until the time point at which it could be measured (4 min of dofetilide infusion, see above); there was no significant difference in the QRS values between the two groups at any time point of the measurement.

3.2.4. Beat-to-beat variability of the QT and the RR interval

The 'sinus' beat-to-beat variability of the RR and QT interval remained stable during the whole experiment in both the 'TdP+' and the 'TdP-' groups; neither 'sinus' RR (Table 5) nor 'sinus' QT variability parameters differed statistically between the two groups at baseline, during the phenylephrine infusion and at the beginning of the dofetilide infusion (Table 6).

When the RR and QT intervals in 40 consecutive cardiac cycles were analysed irrespective of the presence or absence of sinus rhythm during the measurement, a remarkable effect of the drugs was revealed. Phenylephrine infusion increased strikingly all 'real' beat-to-beat RR (Table 5) and QT variability parameters as compared with baseline, which indicates that the drug induced large number of arrhythmias and electrical instability in the heart (Table 6). The 'real' beat-to-beat RR variability values of the 'TdP+' group were slightly but non-significantly higher than the respective values in the 'TdP-' group during phenylephrine infusion (Table 5). Likewise, the 'real' beat-to-beat QT variability values of the 'TdP+' group were slightly but non-significantly higher than the respective values in the 'TdP-' group during phenylephrine infusion (Table 6). Dofetilide administration quickly exaggerated the phenylephrine-induced increase in the 'real' beat-to-beat variability of the RR (Table 5) and QT intervals in both the 'TdP+' and 'TdP-' groups, but the dofetilide-induced increase in these parameters reached statistical significance only in the 'TdP+' group (Table 6).

All kinds of 'real' RR and QT variability parameters increased even further immediately before the occurrence of TdP in the 'TdP+' group showing a continuous, steep increase in these parameters from the start of the phenylephrine infusion till the appearance of TdP during dofetilide administration (Table 5). This indicates that dofetilide quickly and markedly exaggerated phenylephrine-induced arrhythmic activity and electrical instability in the hearts prior to TdP occurrence. Interestingly, the relative increase in the 'real' beat-to-beat

variability of the RR interval before TdP was a magnitude higher than that of the 'real' beat-to-beat variability of the QT interval (Table 6).

		n	MeanRR	RMS	RMSSD	I	TI	STI	STV	LTV
Sinus variability										
TdP-	Basal	10	221±8	221±8	1.8±0.1	2.3±0.3	2.0±0.2	1.2±0.1	1.0±0.1	1.5± 0.1
	Phe15	8	259±14	259±14	2.2±0.2	3.4±0.8	2.8±0.6	1.3±0.1	1.2±0.1	2.2±0.5
	Dof31	8	264±15	264±15	2.4±0.4	3.6±0.8	2.9±0.6	1.3±0.2	1.3±0.2	2.3±0.5
TdP+	Basal	20	215±5	215±2	1.8±0.1	2.5±0.3	2.2±0.2	1.0±0.1	0.9±0.1	1.7±0.2
	Phe15	13	265±11	265±11	3.0±0.7	3.5±0.6	3.5±0.7	1.8±0.4	1.7±0.4	2.6±0.5
	Dof31	12	270±11	270±11	3.6±0.7	4.0±0.6	3.9±0.6	2.1±0.4	2.0±0.4	2.8±0.5
Real variability										
TdP-	Phe15	10	257±11	258±11	19.9±11.9	12.4±7.7	15.0±8.0	10.1±6.3	9.8±6.2	8.0±3.8
	Dof31	10	259±12	260±12	31.8±14.2	32.6±17.7	22.6±11.0	19.7±9.7	19.2±9.5	6.3±4.0
TdP+	Phe15	20	262±7	263±7	39.5±9.2	32.5±8.6	27.5±6.5	24.0±6.1	23.4±6.0	7.6±1.5
	Dof31	20	265±7	269±7#	67.9±10.2	59.7±11.0	48.1±7.5	41.8±6.9#	40.7±6.7	13.4±3.3*
	TdP	20	290±17	300±17#	108.9±9.7#†	95.6±12.7#†	89.0±8.1#†	62.3±6.2#†	60.7±6.1#	46.7±6.0#†

Table 5. The 'sinus' and 'real' beat-to-beat variability of the RR interval in anaesthetized rabbits. The values determined from 40 consecutive QT intervals before drug administration (Basal), in the last minute of the 15 µg/kg/min phenylephrine infusion rate (Phe15), after 4 minute of the beginning of the dofetilide infusion (Dof31) and immediately before TdP (TdP). Group size is indicated by n. All values shown as means ± S.E.M. *P<0.05 vs. 'TdP-'; #P<0.05 vs. Phe15; †P<0.05 vs. Dof31. For further details, see chapter *2.6.4.*

		n	MeanQT	RMS	RMSSD	I	TI	STI	STV	LTV
Sinus variability										
TdP-	Base	10	167±6	168±6	6.5±0.5	7.9±0.7	7.0±0.5	4.1±0.3	3.6±0.3	4.9±0.5
	Phe15	8	185±12	185±12	7.7±0.6	8.6±1.0	7.4±0.6	4.3±0.4	4.2±0.4	4.9±0.4
	Dof31	8	207±10	208±10	9.5±1.2	10.4±1.4	9.5±1.0	5.2±0.7	5.1±0.7	6.7±0.6
TdP+	Basal	20	169±4	169±4	6.3±0.4	7.3±0.7	6.5±0.5	3.5±0.2	3.4±0.2	4.6±0.4
	Phe15	13	192±7	192±7	8.3±0.5	9.2±1.0	8.7±0.6	4.7±0.3	4.6±0.3	6.2±0.7
	Dof31	12	193±7	193±7	8.5±1.1	9.8±1.4	8.8±1.0	4.7±0.5	4.6±0.5	6.3±0.8
Real variability										
TdP-	Phe15	10	190±9	190±9	12.1±2.6	11.8±2.5	10.8±2.0	6.7±1.5	6.5±1.5	6.9±1.2
	Dof31	10	190±6	191±6	21.7±7.0	20.6±7.2	17.3±4.6	13.0±4.8	12.7±4.7	8.2±1.0
TdP+	Phe15	20	195±5	195±5	16.3±2.6	14.3±2.5	13.5±1.8	9.5±1.7	9.3±1.7	7.5±0.6
	Dof31	20	205±5*	206±5*#	34.4±6.2#	28.6±5.8#	26.8±4.4#	20.5±4.1#	20.0±4.0#	12.6±2.8#
	TdP	20	237±11#†	242±11#†	56.1±5.1#†	50.4±7.1#†	52.6±5.2#†	30.6±2.9#†	29.9±2.8#†	35±4.1#†

Table 6. The 'sinus' and 'real' beat-to-beat variability of the QT interval in anaesthetized rabbits. The values determined from 40 consecutive QT intervals before drug administration (Basal), in the last minute of the 15 µg/kg/min phenylephrine infusion rate (Phe15), after 4 minute of the beginning of the dofetilide infusion (Dof31) and immediately before TdP (TdP). Group size is indicated by n. All values shown as means ± S.E.M. *P<0.05 vs. 'TdP-'; #P<0.05 vs. Phe15; †P<0.05 vs. Dof31. For further details, see chapter *2.6.4.*

3.2.5. Baroreflex sensitivity and the spectral power of the systolic arterial pressure and the RR interval

The mean up-baroreflex sensitivity (up-BRS) was slightly, but non-significantly higher at all predetermined time points in the 'TdP+' group as compared with the respective values in the 'TdP-' negative group (data not shown). However, when only a single between-group analysis was performed using the pooled baroreflex sensitivity data on all five 1-min time periods during the phenylephrine infusion, a significantly higher up-BRS was found in the 'TdP+' group than in the 'TdP-' group. The up-BRS values were 2.27±0.14 and 1.94±0.24 ms/(mm Hg) in 'TdP+' and 'TdP-' group, respectively.

The down-BRS did not change during the phenyleprhine infusion in either group as compared with the baseline values; there was no statistical difference in the down-BRS between the 'TdP+' and the 'TdP-' groups at any time point of the measurement (data not shown).

The mid-frequency spectral power of the systolic arterial pressure, which is a good marker of the sympathetic activity, decreased slightly during phenylephrine infusion as compared with the baseline values [10.1±4.8 $(mm\ Hg)^2$ and 7.4±2.7 $(mm\ Hg)^2$ at baseline versus 6.4±3.0 $(mm\ Hg)^2$ and 3.7±1.6 $(mm\ Hg)^2$ during phenylephrine in the 'TdP+' and 'TdP-' group, respectively]. There was no statistical difference in the respective values between the 'TdP+' and 'TdP-' groups at baseline and during phenylephrine infusion.

3.2.6. Blood gases and ion levels

The blood gases and the serum levels of K^+, Na^+ and Ca^{2+} were in the physiological range at baseline, during the phenylephrine infusion and at the beginning of the dofetilide infusion; there were no statistical differences in the serum levels of K^+, Na^+ or Ca^{2+}, or the serum pH^+ or blood gas values between the two groups at baseline, during the phenylephrine infusion and at the beginning of the dofetilide infusion (data not shown).

3.3. Examination of the role of the Na^+/Ca^{2+} exchanger, I_{Na} and I_{CaL} in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts

3.3.1. The effect of AV block on the ventricular rhythm and repolarization

As expected, the applied mechanical AV block decreased the average ventricular heart rate (Figure 3). Interestingly, AV ablation also led to a chaotic and irregular spontaneous ventricular rhythm (Figure 3b), and it strikingly increased all parameters of the beat-to-beat

variability of the RR intervals (Figure 3d). In addition, the AV ablation increased all QT variability parameters, which reflected a tentative beat-to-beat repolarization (Figure 3c).

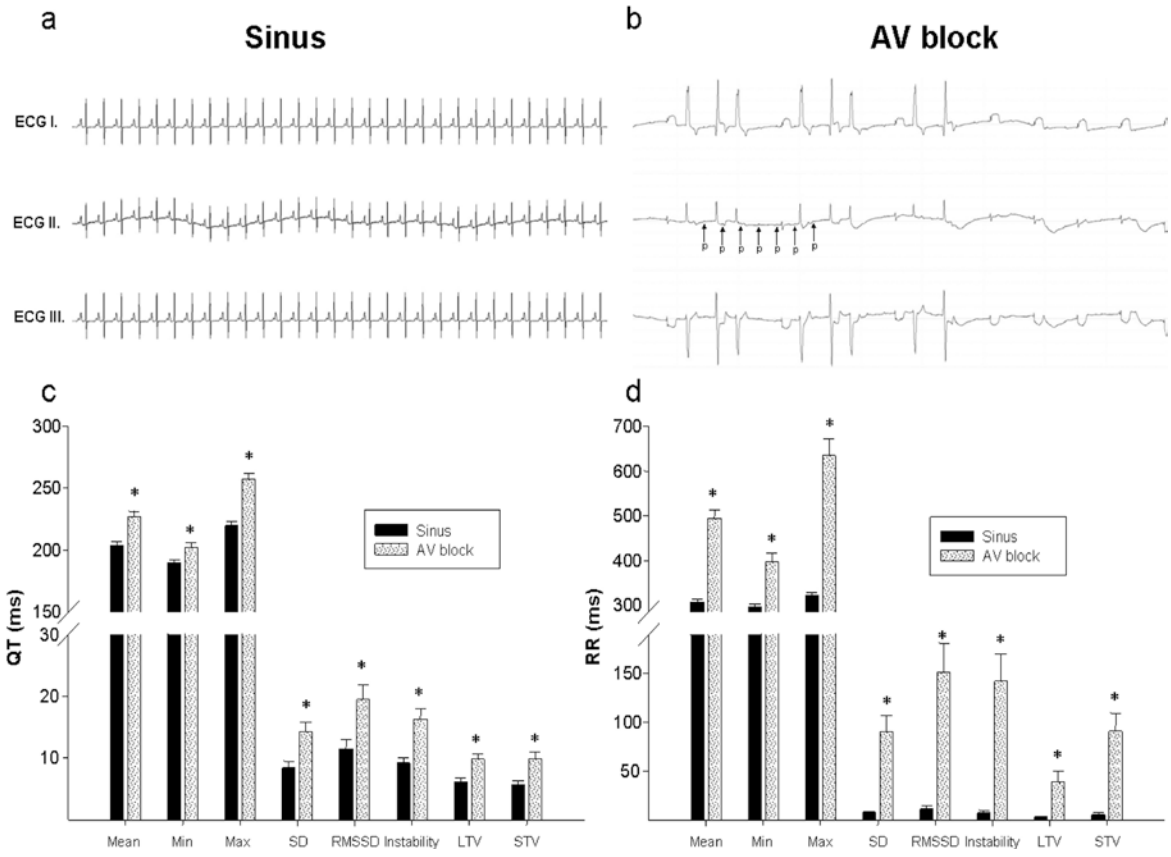

Figure 3. Representative ECG recordings during sinus rhythm (a) and after AV ablation (b). Beat-to-beat variability parameters of the QT (c) and the RR (d) intervals during sinus rhythm and after AV ablation in isolated rabbit hearts. Vertical arrows indicate P waves. ECG I-III, electrocardiographic leads similar to standard limb leads I, II and III; Sinus, sinus rhythm; AV block, chaotic idioventricular escape rhythm after AV ablation; Mean, the mean of the measured 40 consecutive QT or RR intervals (ms); Min: the minimum of the measured 40 consecutive QT or RR intervals (ms); Max, the maximum of the measured 40 consecutive QT or RR intervals (ms); All values shown as means ± S.E.M. *P<0.05 vs. sinus rhythm. For further details, see chapter *2.6.4.*

3.3.2. TdP incidences and onset times

In the first set of experiments, TdP occurred in the majority of the dofetilide-perfused hearts (Table 7). NCX inhibition with SEA0400 did not reduce the incidence of dofetilide-induced TdP (Table 7) and did not affect the onset time of this arrhythmia (325±30 s and 409±97 s after switching to dofetilide perfusion in the DOF1 and SEA+DOF group, respectively). Interestingly, TdP occurred in one heart in the DMSO-perfused control group 1174 s after switching to DMSO perfusion.

In the second set of experiments, dofetilide provoked TdP in the majority of the hearts as seen in the first set of experiments. Lidocaine significantly decreased the incidence of dofetilide-induced TdP, while verapamil completely prevented the development of this arrhythmia (Table 7).

1st set of exp.	**TdP incidence (%)**	2nd set of exp.	**TdP incidence (%)**
H2O	0	**DOF2**	88
DMSO	13	**LID+DOF**	13†
DOF1	75#	**VER+DOF**	0†
SEA+DOF	100*#		

Table 7. Incidence of torsades de pointes (TdP) in AV-ablated, isolated rabbit hearts. The hearts were perfused with distilled water (H_2O), solvent of dofetilide and SEA0400 (DMSO), 100 nM dofetilide (DOF1) or 1.0 μM SEA0400 and 100 nM dofetilide (SEA+DOF) in 1st set of experiments. Hearts were perfused with 100 nM dofetilide (DOF2), 30 μM lidocaine and 100 nM dofetilide (LID+DOF) or 750 nM verapamil and 100 nM dofetilide (VER+DOF) in the 2nd set of experiments. Each group contained eight (n=8) hearts. Values are percentage incidences of arrhythmias. *P<0.05 vs. DMSO, #P<0.05 vs. H_2O, †P<0.05 vs. DOF2.

3.3.3. The RR and the QTc intervals and the beat-to-beat variability of the RR and QT intervals

During the 'pretreatment period', in the first set of experiments neither DMSO nor SEA0400 affected the mean RR interval (Table 8), the mean QTc interval (Table 9) or any of the RR or QT variability parameters (Table 8, Table 9). After the exclusion of all hearts that experienced asystole longer than 20 s (as a predetermined exclusion criteria), the mean RR interval, the mean QTc interval or any of the RR or QT variability parameters did not significantly differ between the lidocaine, verapamil and the control groups before dofetilide perfusion (Table 8, Table 9).

During 'treatment period', in the 1st set of experiments, dofetilide perfusion significantly increased the mean QTc intervals (Table 9) and the beat-to-beat variability of the QT interval (Table 9) without affecting the mean RR interval (Table 8) and the beat-to-beat variability of the RR interval (Table 8). NCX inhibition with SEA0400 exaggerated the dofetilide-induced increase in the mean QTc interval (Table 9) and further increased some of the QT variability parameters (Table 9). Further, SEA0400 upon dofetilide perfusion increased the beat-to beat variability of the RR interval similarly to QT variability parameters without affecting the mean RR interval (Table 8).

Verapamil on top of dofetilide perfusion significantly increased the mean RR interval i.e. decreased the ventricular heart rate as compared with that of the DOF2 group (Table 8). Verapamil and lidocaine tended to increase the beat-to-beat variability of the RR interval but

only the RR RMS parameter of the VER+DOF group reached statistical significance as compared with the DOF2 group (Table 8).

		meanRR	SD	RMS	RMSSD	I	LTV	STV
1st set of experiments								
H_2O	Basal	441±85	53±20	447±65	92±37	71±35	21±9	55±24
	Pretreat	854±101	36±14	681±147	44±19	45±21	26±11	24±12
	Treat	1427±676	174±145	783±197	191±162	44±21	105±80	48±32
DMSO	Basal	472±65	63±35	482±67	81±56	116±68	37±30	50±38
	Pretreat	809±190	385±211	944±258	492±254	208±66	260±157	168±69
	Treat	662±167	135±57	680±174	143±41	223±91	119±65	77±23
DOF1	Basal	438±25	42±20	443±24	72±38	57±36	17±9	44±25
	Pretreat	515±72	117±93	566±88	171±140	51±35	52±37	58±46
	Treat	869±291	398±216	1057±318	535±313	166±90	231±94	149±62
SEA+ DOF	Basal	542±26	151±48	575±29	251±91	281±85	68±30	151±56
	Pretreat	725±137	52±26	729±137	34±18	48±21	45±21	14±7
	Treat	1146±72	429±109*#	1251±81	534±152	350±82#†	341±90	244±70
2nd set of experiments								
DOF2	Basal	508±27	141±54	541±38	262±99	232±87	39±34	165±62
	Pretreat	577±59	75±43	590±63	113±79	102±83	37±18	65±53
	Treat	661±86	183±49	706±75	267±76	202±72	120±33	125±34
LID+ DOF	Basal	557±63	108±40	574±66	180±81	169±82	36±11	112±57
	Pretreat	854±101	155±107	892±122	268±208	279±202	37±30	171±140
	Treat	1427±676	649±321	1660±716	1043±505	588±375	333±175	519±267
DOF+ VER	Basal	506±57	89±54	530±61	141±85	112±54	56±44	77±42
	Pretreat	969±182	33±16	970±182	36±17	35±15	27±13	12±5
	Treat	1606±280ǂ	854±3	1920±342ǂ	1301±540	11169±495	540±211	709±304

Table 8. The beat-to-beat variability of the RR interval in AV-ablated, isolated rabbit hearts. The values determined from 40 consecutive QT intervals during baseline (Basal), pretreatment period (Pretreat), treatment period (Treat); Values are mean ± S.E.M. *P<0.05 vs. DMSO; #P<0.05 vs. H_2O; †P<0.05 vs. DOF1; ǂP<0.05 vs. DOF2. For further details, see chapter *2.6.4.*

In the 2nd set of experiments, verapamil further increased the dofetilide-induced QTc prolongation whereas lidocaine did not affect the mean QTc interval significantly when compared with that of the DOF2 group (Table 9). Verapamil tended to increase the beat-to-beat variability of the QT interval but only the QT RMS parameter reached statistical significance. Similarly, lidocaine on top of dofetilide perfusion significantly elevated QT RMS as compared with that of the DOF2 group (Table 9).

		meanQTc	SD	RMS	RMSSD	I	LTV	STV
1st set of experiments								
H_2O	Basal	230±9	11±2	225±13	15±2	12±2	8±1	8±1
	Pretreat	239±9	11±2	254±6	11±2	14±3	10±3	6±1
	Treat	235±8	14±2	253±19	18±3	14±2*	11±2	9±2
DMSO	Basal	234±10	17±4	233±12	24±7	20±5	11±3	13±4
	Pretreat	226±7	18±4	251±13	23±5	20±3	14±3	12±2
	Treat	253±11	22±3	267±15	30±6	30±5#	16±2	17±4
DOF1	Basal	222±7	14±2	217±9	16±2	17±3	11±2	9±1
	Pretreat	224±4	16±5	226±6	20±5	19±4	13±3	10±2
	Treat	351±40*#	73±15*#	389±55*#	87±17*#	65±19#	63±16*#	40±8*#
SEA+ DOF	Basal	221±10	14±4	225±12	18±6	21±7	12±3	10±4
	Pretreat	239±15	14±2	258±20	18±3	17±3	12±2	9±2
	Treat	524±43*#†	101±27*#	589±45*#†	91±25*#	145±53*#	102±32*#	42±12*#
2nd set of experiments								
DOF2	Basal	241±9	15±3	242±10	23±5	21±5	9±1	13±4
	Pretreat	239±8	12±2	245±9	17±4	14±3	9±2	9±2
	Treat	291±17	42±12	308±17	49±14	60±16	35±12	27±8
LID+ DOF	Basal	222±10	18±8	228±15	26±18	11±2	10±3	9±2
	Pretreat	225±12	21±12	256±14	31±18	17±5	17±10	15±9
	Treat	383±45	31±9	459±97ǂ	47±14	45±15	20±6	27±8
VER+ DOF	Basal	224±7	12±2	225±8	16±3	12±2	8±1	9±2
	Pretreat	254±10	19±6	293±17	22±5	17±3	13±3	10±1
	Treat	498±90ǂ	101±36	588±115ǂ	135±61	161±62	72±23	78±39

Table 9. The beat-to-beat variability of the QT interval in AV-ablated, isolated rabbit hearts. The values determined from 40 consecutive QT intervals during baseline (Basal), pretreatment period (Pretreat), treatment period (Treat); Values are mean ± S.E.M. *P<0.05 vs. DMSO; #P<0.05 vs. H_2O; †P<0.05 vs. DOF1; ǂP<0.05 vs. DOF2. For further details, see chapter *2.6.4.*

3.4. Analysis of the effect of Na^+/Ca^{2+} exchanger inhibition on the cardiac muscle contractility in isolated rat and rabbit hearts

3.4.1. Effects of SEA0400 on $I_{Na/Ca}$ in isolated myocytes

SEA0400 suppressed both the inward and the outward $I_{Na/Ca}$. IC_{50} values of the inward (forward) and the outward (reverse) currents were 243 nM and 309 nM in rabbit (Figure 4a,c), and 120 nM and 61 nM in rat myocytes, respectively. No significant difference was seen in the SEA0400-induced suppression of the inward and the outward $I_{Na/Ca}$. (Figure 4b,d).

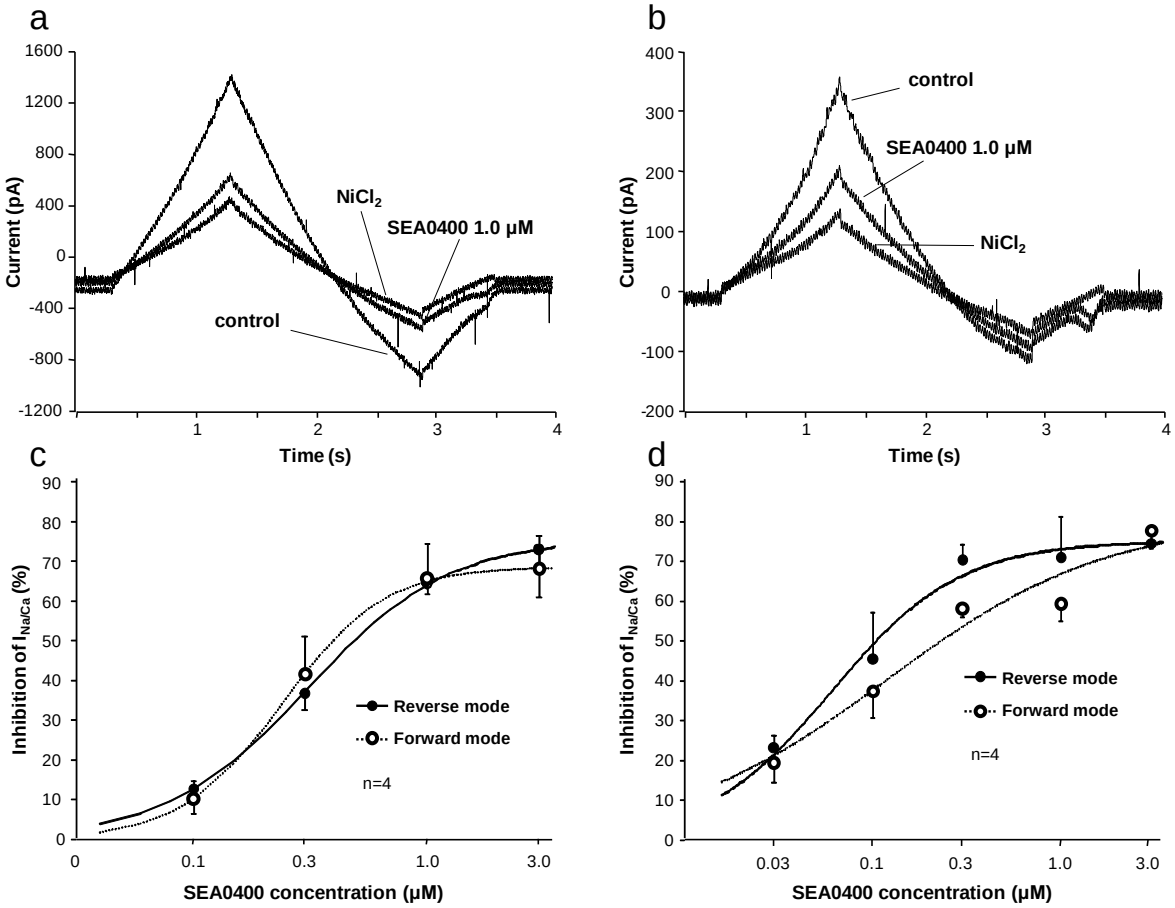

Figure 4. Concentration-dependent effects of SEA0400 on $I_{Na/Ca}$ in isolated rabbit and rat myocytes. Superimposed current traces obtained in K^+-free bath solution (control), in the presence of 1.0 µM SEA0400 and after addition of 10 mM $NiCl_2$ in order to fully block $I_{Na/Ca}$ in the rabbit (a) and the rat myocyte (b). Outward and inward $I_{Na/Ca}$ were determined during the descending limb of the ramp at +40 and –80 mV. Concentration-dependent blocking effect of SEA0400 on forward $I_{Na/Ca}$ (open symbols) and reverse $I_{Na/Ca}$ (filled symbols) in rabbit (c) and in rat (d) myocytes. The solid line was obtained by fitting data to the Hill equation. Four cells were challenged in every examined concentrations of SEA0400. Symbols and bars represent mean±S.E.M

3.4.2. Systolic, end-diastolic and developed pressures

Rabbit

During the first Starling curve and at a constant balloon volume (Krebs solution), the applied balloon stretch increased the systolic and developed pressures very similarly in all four groups. The SEA0400 concentrations applied (0.1, 0.3 and 1.0 µM) failed to alter the systolic and developed pressures at the constant balloon volume and in the second Starling curve as compared with the control group (Table 10). When the systolic pressures measured at the maximum balloon volume of the second Starling curve were divided by the corresponding values of the drug-free first Starling curve, a concentration-dependent, but slight and insignificant positive inotropic effect of SEA0400 was revealed (Figure 5a). A

similar tendency was observed in the corresponding values of the developed pressure. The diastolic pressure was not changed significantly during the second Starling curve and the developed pressure did not differ between the groups during perfusion with SEA0400 (Table 10).

a	Ball V		0.5	0.1	0.3	0.5	0.7	0.9	1.1	1.3	1.5
Systolic P	Control		100±9	78±7	89±7	98±9	106±10	114±10	122±10	128±10	128±12
	0.1	SEA0400	98±7	77±10	88±9	95±7	102±7	109±6	116±7	124±7	134±7
	0.3		92±9	70±11	81±10	90±10	101±10	109±9	120±11	126±9	129±8
	1.0		111±7	91±7	97±7	106±7	114±7	120±4	129±8	136±8	141±7
Diastolic P	Control		1,3±0,6	-3,2±1,3	0,6±0,7	1,6±0,7	2,8±0,7	3,7±0,7	4,5±0,9	5,5±0,9	6,0±1,4
	0.1	SEA0400	0,6±0,4	-2,8±0,8	1,2±0,5	1,2±0,4	1,9±0,4	2,4±0,5	3,1±0,6	4,1±0,9	5,5±1,2
	0.3		0,9±0,6	-2,9±0,6	0,1±0,6	1,1±0,6	2,6±0,5	3,8±0,8	4,1±0,7	4,0±0,8	4,9±1,0
	1.0		1,7±0,4	-2,5±0,4	0,9±0,4	1,7±0,4	2,4±0,4	3,5±0,5	4,7±0,6	5,8±0,9	6,9±1,3
Dev P	Control		99±9	81±7	89±7	96±9	103±10	110±10	117±10	122±9	122±11
	0.1	SEA0400	98±6	79±9	89±8	94±6	100±6	106±6	113±6	120±6	129±7
	0.3		91±9	73±11	91±10	89±9	98±9	105±9	115±11	122±8	108±17
	1.0		109±6	93±7	96±7	104±6	111±7	117±7	125±7	131±8	134±7

b	Ball V		0.08	0.02	0.04	0.06	0.08	0.10	0.12	0.14	0.16	0.18
Systolic P	Control		77±3	35±3	53±2	68±3	83±3	97±3	109±3	118±3	127±3	135±4
	0.1	SEA0400	85±3	33±3	58±3	74±3	91±3	103±3	112±4	124±4	127±6	138±5
	0.3		84±4	38±4	60±4	77±4	91±4	104±4	115±3	125±4	134±3	146±4
	1.0		90±4*	38±2*	64±3*	84±4*	100±5*	115±6*	126±6*	136±7*	146±7*	153±8*
Diastolic P	Control		-0,1±0,6	-8,3±0,9	-2,4±0,7	-0,8±0,6	0,4±0,6	1,3±0,6	2,1±0,6	3,4±0,6	4,8±0,5	6,7±0,5
	0.1	SEA0400	-0,1±0,6	-10,9±1,2	-2,2±0,7	-0,6±0,6	0,8±0,7	1,4±0,7	2,5±0,8	4,1±0,9	3,9±0,7	6,0±1,1
	0.3		0,1±0,3	-8,7±0,6	-2,3±0,2	-0,5±0,2	0,8±0,3	1,8±0,3	2,4±0,4	3,0±0,3	4,0±0,3	5,4±0,4
	1.0		0,6±0,3	-11,2±0,6	-2,3±0,3	0,0±0,4	1,2±0,4	2,4±0,5	3,2±0,6	4,3±0,9	6,0±1.0	5,8±0,7
Dev P	Control		77±3	43±3	55±2	59±3	82±4	96±4	107±4	115±4	122±3	129±4
	0.1	SEA0400	85±3	43±2	61±3	74±3	91±3	102±3	110±3	120±4	123±6	132±5
	0.3		84±4	47±4	62±4	77±4	90±4	102±4	113±3	122±4	130±3	141±3
	1.0		89±4*	50±2*	66±3*	84±4*	99±4*	112±6*	123±6*	132±6*	140±6*	147±8*

Table 10. Systolic, diastolic and developed pressures in the presence of SEA0400 in the rabbit heart (Part a) and the rat heart (Part b). Systolic P: left ventricular systolic pressure (mm Hg); Diastolic P: left ventricular diastolic pressure (mm Hg); Dev P: developed pressure (systolic minus end-diastolic pressure) (mm Hg). Ball V: volume of the left ventricular balloon (ml). $^{*}P<0.05$ vs. control.

Rat

Following the striking resemblance between the four groups in the drug-free first Starling curve the applied SEA0400 concentrations increased the systolic pressure in a concentration-dependent manner both at a constant balloon volume and during the second Starling curve (Table 10). A significant and concentration-dependent positive effect of SEA0400 was also revealed when the systolic pressures measured at the maximum balloon volume of the second Starling curve were divided by the corresponding values of the drug-free first Starling curve (Figure 5b). The 1.0 μM SEA0400 group differed significantly from the control**.** The developed pressure also increased in a concentration-dependent manner

during perfusion with SEA0400 since the systolic pressure increased whereas the diastolic pressure remained unchanged (Table 10).

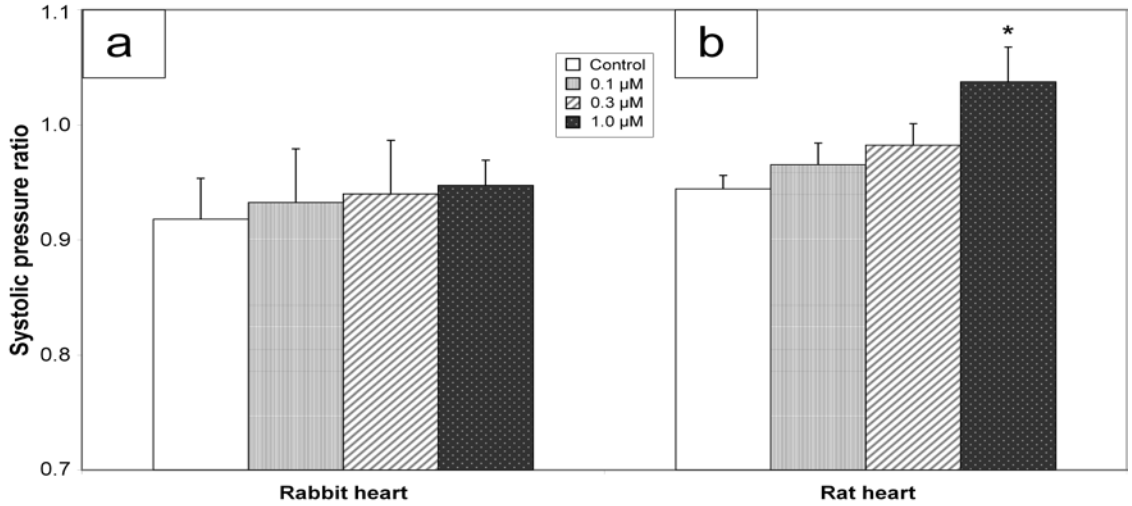

Figure 5. Systolic pressure ratios of the maximum balloon volume of the second (drug-perfused) and the first (drug-free) Starling curves in the isolated rabbit (a) and in the rat heart (b). Values are means ± S.E.M. $^{*}P<0.05$ vs. control.

3.4.3. ECG intervals and heart rate

The ECG intervals were almost constant during both Starling curves in both species. Perfusion with SEA0400 did not influence the PQ, QRS and QT intervals in either species (data not shown). The increased left ventricle stretch did not affect the ECG intervals; no differences were found between the constant and alternating balloon volume periods. SEA0400 perfusion did not exert any effect on the heart rate in either species. The corresponding ECG intervals of the SEA0400-perfused groups of hearts did not differ from those for the control group in either the rabbit or the rat study (data not shown).

3.4.5.NCX protein density

Immunohistochemistry with confocal microscopy imaging revealed similar scattered distributions of the NCX protein on the surface of the rabbit and the rat myocytes (Figure 6). The physiological cardiac sarcolemmal NCX protein density was 88.0±2.7 and 82.5±2.3 in the rabbit and the rat, respectively. There is no difference between the NCX protein density of the rabbit and the rat.

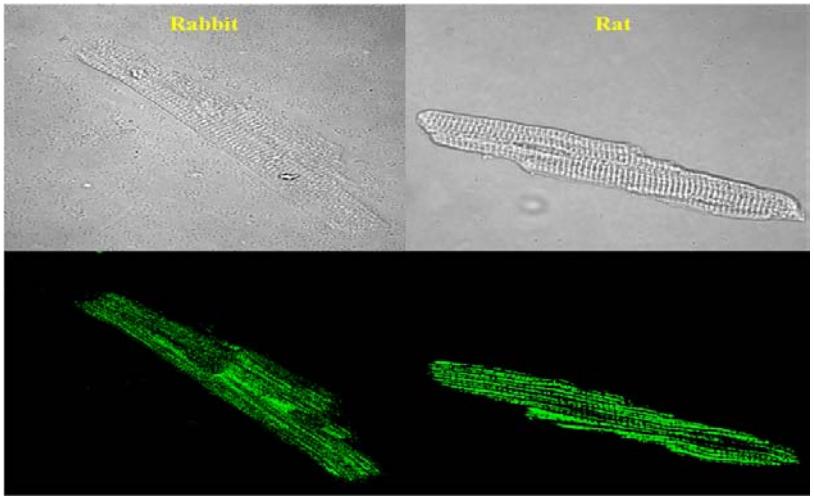

Figure 6. Native and NCX immunofluorescent confocal laser scanning microscope images of the rabbit myocytes and the rat

4. DISCUSSION

4.1. The effect of α_1-adrenoceptor stimulation and the constant left ventricular stretch on the development of torsades de pointes in isolated rabbit hearts

The present results of the 'i*n vitro* α_1-adrenoceptor stimulation and stretch study' indicate that neither a sustained load-induced constant high level of left ventricular stretch nor α_1-adrenoceptor stimulation nor their co-application, promote the generation of TdP in the setting of prolonged repolarization in isolated, Langendorff-perfused rabbit hearts. The roles of intracardiac α_1-adrenoceptor stimulation and a sustained load-induced left ventricular stretch in the provocation of TdP in the rabbit are therefore questionable. In contrast, the present results suggest the importance of extracardiac α_1-adrenoceptor stimulation in TdP development in the rabbit heart.

4.1.1. Sufficient dofetilide effect

In the hearts in the present investigation, 100 nM dofetilide markedly and significantly prolonged the QT interval and evoked TdP, which is clear evidence for the efficient repolarization prolongation in the rabbit [8]. Furthermore, 100 nM dofetilide induced conduction blocks in most hearts and widened the QRS complex, which is direct and indirect evidence, respectively, of extremely prolonged repolarization and refractoriness in the conduction system of the rabbit, which occurs when high doses of repolarization-prolonging drugs are applied [24]. The presence of conduction blocks in the dofetilide-perfused hearts in this investigation suggests that the conditions were given for functional reentry circuits to

develop as a result of a sufficient dofetilide effect. This supports the notion that the dofetilide concentration was high enough to set the stage for the development of TdP in this study.

Despite the sufficient effect of 100 nM dofetilide on repolarization and refractoriness, TdP occurred in fewer than half of the hearts in the first set of experiments. A zero incidence of this arrhythmia was earlier seen with 100 nM dofetilide in isolated Langendorff-perfused rabbit hearts [40], though that perfusion solution did not differ significantly from the one used in the present study. The present incidence of TdP was enough to provide scope for the examination of additional effects that might increase the incidence of TdP.

4.1.2. Stretch did not initiate TdP

In the present experiments a constant high level of stretch did not increase the incidence of dofetilide-induced TdP, though the stretch was arrhythmogenic since it increased the incidence of salvo and of ventricular tachycardia different from TdP, and ventricular premature beats occurred in each heart only in those two groups, in which the stretch was applied. In theory, these stretch-induced arrhythmias could initiate TdP in the presence of dofetilide-produced functional reentry circuits. One possible explanation of the lack of a stretch effect on TdP initiation is that the absolute number of stretch arrhythmias was still low, and the statistical possibility of a stretch-induced arrhythmia falling in the vulnerable period of dofetilide-produced functional reentries was therefore low. Thus, further elevation of the absolute number of stretch-induced arrhythmias might increase the incidence of dofetilide-induced TdP. In the present experiments, the left ventricular stretch applied was the highest that did not destroy the structure of the ventricle and provided physiological systolic and end-diastolic pressures. Elevation of the absolute number of stretch-induced arrhythmias would therefore be possible only by overstretching the left ventricle with non-physiological volumes [41] or by applying a dynamically changing stretch protocol with rapid volume pulses [42]. However, the application of such procedures was not the aim of the study.

It is known that a stretch can shorten repolarization. In an earlier study, a sustained load produced by a filled left ventricular balloon shortened the duration of the monophasic action potential in isolated, Langendorff-perfused rabbit hearts [43]. In guinea pig myocytes, a hyopotonic-induced stretch shortened the action potential duration and counteracted the effect of the potent repolarization-prolonging agent E4031 [44]. In the present study, the QT interval was not affected by the stretch, which implies that the stretch did not counteract the repolarization-prolonging effect of dofetilide.

A ventricular stretch produces complex electrophysiological effects by inducing afterdepolarizations, increasing the dispersion of repolarization, slowing impulse conduction, and either shortening or prolonging repolarization [11, 45], which may affect the genesis of TdP. However, no data have so far been published on the contribution of a ventricular stretch to the development of TdP. Accordingly, despite the fact that the present study did not reveal any effect of a sustained load-induced stretch on the occurrence of TdP in isolated rabbit hearts perfused with dofetilide, the roles of a ventricular stretch and stretch-induced complex electrophysiological changes in the generation of TdP necessitate further investigations in other experimental settings.

4.1.3. Methoxamine did not promote TdP in vitro

In the anaesthetized rabbit model of acquired long QT syndrome [8], the stimulation of α_1-adrenoceptors by either methoxamine [8] or phenylephrine [10] sensitizes the animals to the proarrhythmic effects of repolarization-prolonging drugs. Stimulation of the cardiac α_1-adrenoceptors increases the free intracellular Ca^{2+} level via an increased turnover of phosphatidylinositols [46], suppresses outward K^+ currents [47-51] and increases the amplitude of EADSs [52], all of which may contribute to the development of TdP in the setting of prolonged repolarization.

Interestingly, methoxamine did not promote TdP generation in rabbit hearts in the present study. The currently applied *in vitro* concentration of methoxamine (100 nM) may not match the *in vivo* serum concentration of the drug achieved by continuous venous infusion (usually at a rate of 15 μg/kg/min). However, the minimum concentrations of methoxamine that cause contraction of the pulmonary arteries [53], the ear arteries [54] and the thoracic aorta [55] in the rabbit are 30 nM, 10-30 nM and 100 nM, respectively, which indicates that 100 nM methoxamine does exert pharmacological action in the rabbit. Thus, this concentration seems appropriate for testing the effects of α_1-adrenoceptor stimulation on TdP genesis *in vitro*.

Similarly to our results, it was earlier found that methoxamine at a concentration of 30 nM, which is approximately one-third of that used in the present experiments (100 nM), did not help dofetilide (100-700 nM) to elicit TdP consistently in isolated, Langendorff-perfused rabbit hearts [40]. In that work, repolarization-prolonging drug-induced TdP occurred frequently when acetylcholine (300 nM) was added to the methoxamine in the perfusion solution [40]. Their results and those of the present investigation rather suggest that the role of the stimulation of intracardiac α_1-adrenoceptors in the generation of TdP in the

rabbit is questionable. Furthermore, in the setting of prolonged repolarization, the lack of a sensitizing effect of α_1-adrenoceptor stimulation in isolated rabbit hearts and the presence of this effect in anaesthetized rabbits implies that the extracardiac effects of α_1-adrenoceptor stimulation are the crucial factors in the genesis of TdP.

4.2. The endogenous factors and predictive parameters of dofetilide-induced torsades de pointes in α_1-adrenoceptor stimulated, anaesthetized rabbits

The results show that the endogenous factors and surrogate parameters of dofetilide-induced TdP in pentobarbital-anesthetized, α_1-adrenoceptor stimulated rabbits. Dofetilide-induced TdP was not determined by the baseline haemodynamics, repolarization properties, autonomic status, blood gases or serum ion concentrations. However, those animals that subsequently experienced TdP had more responsive baroreflex during phenylephrine infusion before dofetilide administration. When measured in sinus rhythm, none of the repolarization-related parameters forecasted dofetilide-induced TdP at any stage of the experiment. However, when arrhythmic activity was involved in the measurement of the beat-to-beat variability of the RR and QT interval, 'real' variability of the RR and QT intervals predicted subsequent TdP occurrence.

4.2.1. QT prolongation is necessary but not sufficient for TdP in the rabbit

To date, despite widespread use of the α_1-adrenoceptor-stimulated, anaesthetized rabbit model of TdP [56], drugs that do not prolong the QT interval have not been reported to induce TdP in the model. This suggests that the negative predictive value of the length of the QT interval is high in rabbits. On the other hand, QT prolongation did not necessary result in TdP in the model [9, 28, 57, 58]. Accordingly, when QT interval was measured strictly in sinus rhythm in the present study, we found that dofetilide prolonged the QT interval equally in all animals, but did not induce TdP in all of them. These results show that QT prolongation is necessary but not sufficient for the generation of TdP in α_1-adrenoceptor-stimulated, anaesthetized rabbits. This is in a good accordance with results obtained from other experimental and clinical studies showing that the prolongation of the QT interval alone is insufficient to directly initiate arrhythmia [2, 56].

4.2.2. Only 'real' beat-to-beat variability of the RR and QT interval predict TdP in α_1-adrenoceptor-stimulated, anaesthetized rabbits

Dynamic ECG beat-to-beat variability parameters, such as instability or short term variability, have been proposed recently as better surrogates than QT interval for TdP [2, 16]. Short term variability of the QT interval (STV_{QT}), was a successful predictive marker in sotalol-induced proarrhythmia in chronically AV-blocked dogs in vivo [2]. STV_{QT} increased in patients with an increased risk of arrhythmia in the context of latent repolarization disorder as well as in drug induced long QT syndrome, while QTc did not change [59]. Likewise, an increase in STV_{QT}, when measured in sinus rhythm ('sinus' STV_{QT}), predicted drug-induced TdP occurrence in anaesthetized rabbits without α_1-adrenergic stimulation [60]. In contrast, dofetilide did not increase 'sinus' STV_{QT} and the parameter did not predict TdP in our study with α_1-adrenoceptor-stimulated, anaesthetized rabbits, which is in a good agreement with the results of all earlier studies utilising the same model [18, 28, 61]. Thus, beat-to-beat variability of the QT interval fails to predict TdP, when it is measured in sinus rhythm in the α_1-adrenoceptor-stimulated, anaesthetized rabbit model of TdP.

Interestingly, when arrhythmic activity was involved in the measurement of the beat-to-beat variability parameters, both the 'real' beat-to-beat variability of the RR and QT interval predicted TdP. Dofetilide increased significantly the 'real' beat-to-beat RR and QT variability parameters only in the 'TdP+' group shortly after the start of the infusion, and all of these parameters increased even further before the occurrence of TdP. The simultaneous and progressive increase in the beat-to-beat variability of the RR and QT intervals in the 'TdP+' group implies that there was a progressively increasing arrhythmic activity and electrical instability in the hearts prior TdP occurrence, which may be a prerequisite for the development of TdP in the model.

4.2.3. Vagal nerve activity contributes to drug-induced TdP in rabbits

In the present study, phenylephrine infusion increased significantly the up-BRS in all of the animals before dofetilide infusion, but the increase was significantly higher in the 'TdP+' group. This indicates that the vagal nerve (and the baroreflex) was more responsive to the rise in blood pressure in the animals experiencing TdP than that in the animals not developing TdP. Similarly, two earlier study emphasized the importance of vagal nerve activity in the development of drug-induced TdP. Farkas et al. reported that vagotomy prevented clofilium-induced TdP in α_1-adrenoceptor-stimulated, pentobarbital anaesthetized rabbits [18]. Likewise, inhalation of formaldehyde vapour initiated TdP in conscious,

dofetilide treated rabbits with a mechanism involving reflex co-activation of the parasympathetic and the sympathetic outflows to the heart [62].

Phenylephrine induces bradycardia via activation of the baroreflex resulting in an increase in vagal nerve activity and a reduction in cardiac sympathetic activity in pentobarbital anaesthetized rabbits [18]. Although bradycardia makes the heart susceptible to drug-induced TdP, the phenylephrine-induced bradycardia is not the key factor for TdP induction, since heart rate did not differ between the 'TdP+' and the 'TdP-' groups. Vagal innervation of the ventricles is sparse but ventricular cells do respond to acetylcholine and display regional variations in responsiveness, possibly as a result of variations in the expression of the channels carrying the acetylcholine-activated K^+ current (I_{KAch}) [63, 64]. Vagal nerve activation exerts a significant effect on ventricular repolarization in the rabbit heart by reversing the repolarisation pattern of the left ventricle [65], which may add to dofetilide-induced spatial dispersion of the repolarization leading to the development of re-entry circuits. Acetylcholine can shorten ventricular repolarization by the activation of the I_{KAch} and the inward rectifying K^+ current (I_{K1}) [64, 66]. Regional shortening of the repolarization may help dofetilide-induced EADs to be conducted to the neighbouring cells and develop triggered activity e.g. ventricular premature beats, which can initiate re-entrant arrhythmias e.g. TdP.

4.2.4. Sympathetic activity and TdP

It has been shown that increasing arterial blood pressure with phenylephrine caused a baroreflex-mediated inhibition of sympathetic activity in anaesthetized rabbits [18] In the present study, the mid-frequency spectral power or the systolic arterial pressure, which is a good marker of the sympathetic activity, was reduced slightly by the phenylephrine infusion and did not differ between the 'TdP+' and the 'TdP-' groups at baseline and during the 3rd step of the phenylephrine infusion (9 μg/kg/min), when it was determined. This indicates that the sympathetic activity did not differ between the 'TdP+' and the 'TdP-' groups at baseline and was suppressed by the phenylephrine infusion equally in both groups. This would suggest that the level of the sympathetic activity does not determine the occurrence of dofetilide-induced TdP. However, it is widely accepted that an increased sympathetic activity may contribute to the genesis of TdP [67]. Sympathetic activation increases spatial dispersion of repolarization by unequally shortening the repolarization in the ventricular muscle and also induces triggered activities [65, 67], which can augment the proarrhythmic activity of the repolarization-prolonging drugs. The sympathetic activity of the animals in our study was

estimated only in sinus rhythm, before arrhythmias occurred, as the method required. However, it has been reported, that premature ventricular beats were consistently followed by a burst of sympathetic nerve activation, whereas the postextrasystolic beats were followed by complete neural silence in humans [68]. If premature beats cause fluctuation in the sympathetic activation, then it further increases the electrical instability by creating temporal dispersion of the repolarization. The progressive increase in the beat-to-beat variability of the RR interval during dofetilide infusion shows that the number of arrhythmic beats and the irregularity of the ventricular rhythm progressively increased in the 'TdP+' group prior to TdP occurrence. Recently, it has been shown that greater degrees of the irregularity of the ventricular rhythm caused greater sympathoexcitation independently from the haemodynamic changes [69]. Since premature ventricular beats occurred frequently and the irregularity of the ventricular rhythm progressively increased in the animals before TdP, we cannot exclude that sympathetic activity contributed to dofetilide-induced TdP in our study.

4.2.5. Proposed mechanism of TdP in anaesthetized rabbits

Only a few VPBs developed at the beginning of the experiments due to phenylephrine or dofetilide. However, the sudden change in blood pressure caused by a VPB-activated baroreflex, which altered the parasympathetic and sympathetic activation of the heart. A change in the autonomic outflows resulted in an alteration of spatial dispersion of repolarization and also caused temporal dispersion of repolarization in the ventricles [65]. As a result, other non-complex, 'preceding' arrhythmias developed, which further exacerbated the electrical instability directly and indirectly via further alteration of the autonomic outflows to the heart, and so on. The whole process progressed further and finally produced enough trigger beats and functional re-entry circuits to allow initiation and maintenance of re-entrant arrhythmias e.g. TdP. The progressive increase in the beat-to-beat variability of the RR and QT intervals in our study implies that the process of TdP genesis in α1-adrenoceptor stimulated, anaesthetized rabbits may be described by the chaos theory and non-linear dynamics, a model frequently used to examine the development and surrogates of ventricular tachycardia and fibrillation [70, 71]. The incidence of dofetilide-induced TdP was probably determined by the infusion rate (and the dose) of dofetilide [57], but the individual animal subsequently experiencing TdP was determined only by chance via experiencing slightly more non-complex 'preceding' arrhythmias in number and/or irregularity at the early stage of the experiment, which initiated a dynamic exacerbation of the ventricular inhomogeneity necessary for TdP development.

4.3. The effect of the inhibition of Na^+/Ca^{2+} exchanger on the development of torsades de pointes in isolated rabbit hearts

The 'NCX arrhythmia study' is the first that examined the role of NCX in the genesis of drug-induced TdP. The selective inhibition of NCX by SEA0400 neither decreased the incidence of dofetilide-induced TdP nor influenced the onset time of this arrhythmia in isolated, Langendorff perfused, AV-ablated rabbit hearts. On the other hand, SEA0400 significantly prolonged the mean QTc interval in the presence of dofetilide. The inhibition of the Na^+ channels by lidocaine as well as the block of the L-type Ca^{2+} channels by verapamil significantly antagonized the genesis of dofetilide-induced TdP. However, verapamil further increased the dofetilide-induced QTc prolongation and neither verapamil nor lidocaine reduced the dofetilide-induced increase in the beat to beat variability of the QT interval.

4.3.1. Selective and effective inhibition of NCX in the isolated rabbit heart: no effect on TdP

SEA0400 is probably the most effective inhibitor of the NCX [72], with an inhibitory potency 10 to 100-fold higher than that of KB-R7943 [73]. In isolated rabbit myocardial cells, SEA0400 equally suppressed the inward and the outward NCX current ($I_{Na/Ca}$), IC_{50} values of the inward (forward) and the outward (reverse) currents were 243 and 309 nM, respectively [32]. SEA0400 at a concentration of 1.0 μM inhibits 70-80% of the NCX function [32, 34, 35, 72]. SEA0400 is also considered to be the most selective NCX inhibitor, the drug is considered to be selective for the NCX up to a concentration of 1.0 μM without markedly influencing any other ion transport mechanisms [19, 34].

In a recent study, which was performed on the same Langendorff perfusion rig we used in the present study, SEA0400 at a concentration of 1.0 μM increased significantly the left ventricular contractility in isolated rat hearts, whereas the drug (0.1-1.0 μM) exerted only an insignificant, but concentration-dependent positive inotropic effect in isolated rabbit hearts [32]. Likewise, Szentandrássy *et al*. found that 0.3 and 1.0 μM SEA0400 increased the developed pressure and the amplitude of the Ca^{2+} transient in isolated, Langendorff-perfused rat hearts [74]. These earlier results suggest indirectly that SEA0400 at a concentration of 1 μM blocks effectively the NCX in isolated hearts, too. The effectiveness of verapamil and lidocaine against dofetilide-induced TdP validates our model since it responded to interventions known to be able to reduce the incidence of drug-induced TdP. This suggests that the effective NCX inhibition by SEA0400 did not exert an anti-arrhythmic effect against

dofetilide-induced TdP because NCX does not play a role in TdP genesis in isolated, AV-blocked rabbit hearts.

4.3.2. NCX does not provide the trigger for TdP

According to the most widely accepted theory, TdP is initiated by an EAD-induced ectopic beat [27]. EAD is generated in phase 2 or 3 of the prolonged repolarization as a result of increased depolarising currents such as I_{CaL} and $I_{Na,}$ but also inward NCX current [75]. Stimulation of the β-adrenergic receptors increases I_{CaL} and may result in an overload of the Ca^{2+} stores of the sarcoplasmic reticulum (SR), and a subsequent spontaneous SR Ca^{2+} release can appear as an early Ca^{2+} aftertransient [75]. This activates the forward mode of the NCX leading to EAD generation in guinea pig, canine and human myocytes [76, 77]. Inward NCX current can generate EAD even in the absence of spontaneous SR Ca^{2+} release when K^+ currents are decreased or inhibited [78]. Accordingly, the selective inhibition of NCX with SEA0400 effectively decreased the amplitude of EADs evoked by the co-perfusion of $BaCl_2$ and dofetilide in canine ventricular papillary muscles and Purkinje fibres [19]. On the other hand, acceleration of the pacing rate or a single premature beat could induce EAD activity and APD prolongation in canine ventricular M cell preparations pre-treated with the I_{Kr} blocker E4031 via a mechanism linked to an intracellular calcium-loading-induced electrogenic NCX current [79].

Some authors claim that DAD-induced triggered activity (similarly to EAD) can also initiate TdP [27]. DADs can be produced by Ca^{2+} waves-activated inward currents resulted from the activation of the NCX [75]. Accordingly, NCX inhibition antagonized DAD-dependent digitalis-induced arrhythmias in several in vitro and in vivo experimental models [19, 80-82].

Although plenty of data suggest a role of NCX in the genesis of drug-induced TdP, the selective inhibition of the NCX did not prevent dofetilide-induced TdP in isolated rabbit hearts in the present study. The initiating mechanism of TdP in isolated, Langendorff-perfused, AV-blocked, non-paced rabbit hearts is unknown. However, in an earlier study with AV-blocked rabbit hearts, an EAD was associated with the initiating beat of d-sotalol-induced TdP [83]. If EADs (or DADs) serve as the trigger for TdP in the rabbit hearts in the present study, then the genesis of these triggered activities was not related to the activity of the NCX. It is possible that any beats of the irregular idioventricular escape rhythm that occurred in the vulnerable period of the dofetilide-produced re-entry circuits could serve for a trigger for TdP.

These beats do not depend on NCX activity, which may explain the ineffectiveness of NCX inhibition.

4.3.3. NCX does not contribute to the maintenance of TdP

It is widely accepted that the mechanism of maintenance (substrate) of TdP arrhythmia involves re-entry circuits produced by an increase in spatial dispersion of the repolarization of the ventricular wall. The transmural dispersion has been identified as the principal arrhythmogenic substrate in both acquired and congenital LQTS [27]. The TDR is the consequence of longer APD in M cells compared with the epicardial and endocardial cells in dogs and humans [27]. Zygmunt et al. showed that the longer APD is due to the smaller I_{Ks}, and larger late I_{Na} and NCX currents in the healthy canine ventricle [6]. The I_{Kr} inhibitor d,l-sotalol increased the TDR and induced TdP in isolated, Langendorff-perfused, AV-blocked rabbit hearts [84]. Further, verapamil prevented veratridine-induced TdP via reduction of left ventricular TDR (and suppression of EADs) in isolated, Langendorff-perfused, AV-blocked rabbit hearts [37]. These earlier observations suggest that TDR may also play a role in the maintenance of TdP in AV-ablated rabbit hearts. Although NCX activity contributes to the generation of TDR in the healthy dog heart [6], our results indicate that NCX activity does not increase the TDR and, therefore, does not contribute to the maintenance mechanism of TdP in the rabbit heart.

4.3.4. The effect of SEA0400 on the duration of repolarization

SEA0400 pretreatment did not affect the control QTc, but the drug prolonged significantly the QTc in the presence of the I_{Kr} inhibitor dofetilide. This observation may have two possible explanations. (i) NCX contributes a small outward current at the end of the action potential plateau that, in the absence of repolarization reserve (i.e. in dofetilide) delays repolarization, and/or (ii) SEA0400 is non-selective for NCX and affects other repolarizing currents.

With regard to the former explanation, Sher et al. have reported computer simulations in which NCX may generate an outward current at the end of the action potential plateau [85]. Blocking this current, in the absence of repolarization reserve, may hence prolong action potential duration. However, other simulations by Bers and colleagues [4] have suggested that apart from a very brief period at the start of the action potential NCX remains inward throughout the cardiac cycle. The second possibility is that SEA0400 is not selective for NCX and blocks other repolarizing currents. However, in guinea pig myocytes, SEA0400 at a

concentration of 1.0 μM caused only 4% and 2% inhibition on I_k (at 37 °C) and I_{k1} currents, respectively [34]. SEA0400 may also inhibit smaller repolarizating currents, e.g. I_{Ks}, however it has never been examined. Either explanation requires that the potential repolarization-influencing effect of SEA0400 was minimal when administered alone, but was revealed when the repolarization reserve was reduced by the potent I_{Kr} blocker, dofetilide. Our findings on the effect of SEA0400 on the QTc interval propose that inhibition of the NCX by SEA0400 may exacerbate the proarrhythmic effect of dofetilide. However, this requires further investigation in a setting where control incidence of dofetilide-induced TdP is low.

4.3.5. Neither the duration of the QT interval nor the beat-to-beat variability of the QT interval correlated with the occurrence of TdP

A substantial amount of experimental and clinical data suggests that QT prolongation alone is an unreliable predictor of drug-induced TdP [2]. In our study, verapamil exaggerated the dofetilide-induced QTc prolongation while the drug prevented TdP. Likewise, lidocaine decreased significantly the incidence of TdP but did so without affecting the QTc interval. These results corroborate earlier reports showing that QT prolongation did not correlate to the proarrhythmic liability of repolarization-prolonging drugs in isolated, AV-blocked rabbit hearts [84, 86].

In our isolated heart study, 100 nM dofetilide perfusion increased each QT variability parameter, and the drug induced TdP reproducibly in nearly all hearts. On the other hand, neither verapamil nor lidocaine decreased any of the QT variability parameters, nevertheless they successfully block the development of TdP. Thus, the predictive value of an increase in the beat-to-beat variability of the QT interval in isolated, AV ablated, non-paced rabbit hearts is questionable. Further investigation is needed to clarify whether it is because an increase in the beat-to-beat variability of the action potential is not a contributor to TdP or rather it is because an increase in the beat-to-beat variability of the QT interval is only one out of several contributing factors needed to be present at the same time to allow generation of TdP in the model.

4.3.6. AV block induced electrical instability

In our study, acute AV block was applied in order to slow down the ventricular heart rate since bradycardia has been reported to increase the proarrhythmic activity of I_{Kr} blockers in isolated rabbit hearts [87]. Surprisingly, AV block resulted in a chaotic idioventricular rhythm in all hearts during control conditions. This chaotic idioventricular rhythm is

characterized by an elevated beat-to-beat variability of the RR and QT intervals in our study. Similar electrical instability was reported in isolated mouse hearts after AV ablation, and the level of instability (i.e. the incidence of baseline arrhythmias after AV block) inversely correlated with the K^+ concentration of the perfusion solution [88]. Accordingly, perfusion with 3 mM K^+ induced afterdepolarizations in nearly all mouse hearts and evoked polymorphic ventricular tachycardia in one mouse heart during control conditions, after AV ablation [88]. This may explain why the blinded evaluation of our experiments with 3 mM K^+ in the perfusion solution found a run of 'TdP' in a heart in the DMSO control group. Further, the baseline electrical instability in the AV-blocked rabbit hearts perfused with 3 mM K^+ in the present study might contribute to the high sensitivity of these hearts to the proarrhythmic activity of dofetilide.

4.4. NCX inhibition influences the cardiac contractile force

Our results demonstrate that selective inhibition of the NCX by SEA0400, which is considered as a selective inhibitor of the NCX [34, 72, 89], did not considerably influence the contractility in the isolated rabbit heart, whereas it increased the systolic and developed pressures in a concentration-dependent manner in the isolated rat heart. The NCX inhibition with the selective inhibitor SEA0400 was almost identical in the rabbit and the rat myocytes, when the highest concentration of the drug was used, which significantly increased the contractile force in rats. The immunohistochemistry did not indicate a significant difference between the sarcolemmal NCX protein densities of the rabbit and the rat. Furthermore, heart perfusion with the NCX inhibitor SEA0400 did not influence the ECG intervals (PQ, QRS, QT and RR) or the coronary flow, the latter revealing that the drug did not affect the cardiac vessels either.

4.4.1. Pharmacodynamic effects of SEA0400

Birinyi et al. found that SEA0400 at a concentration of 1.0 μM inhibited the I_{CaL} in canine myocytes, which may explain the lack of positive contractility response in the rabbit heart [35]. Recently, we found in patch clamped rat myocytes that SEA0400-induced increase in the Ca^{2+} transient and cell shortening was accompanied by significant reduction of I_{CaL} [33]. These effects can be explained by the autoregulatory nature of cardiac Ca^{2+} handling, as the reduced Ca^{2+} efflux from the cell results in an increased Ca^{2+} load to the sarcoplasmic reticulum leading to increased Ca^{2+} release, which in turn may decrease the I_{CaL} by

acceleration of Ca^{2+} dependent inactivation of I_{CaL} [33]. Nagy et al. reported that SEA0400 at a concentration of 1.0 μM failed to change I_{CaL} significantly in canine myocytes [19]. Similarly, our data did not reveal any effect of SEA0400 on the ECG PQ intervals, which would have been widened if I_{CaL} had been blocked by the drug [90]. In a recent study, Tanaka et al. [34] measured slightly lower EC_{50} (IC_{50}) values (40 nM and 32 nM for the inward and the outward NCX current, respectively) of the SEA0400 for the NCX in guinea pig myocytes as compared with those we measured in isolated rat and rabbit myocytes. Either the different species or the different experimental circumstances may explain this difference between the results of the present and earlier report. In the present study, SEA0400 (0.03-3.0 μM) inhibited both the forward and the reverse modes of the NCX operation, with similar efficacy, either in isolated rabbit or rat myocytes. SEA0400 had a slightly but non-significantly greater affinity to NCX in the rat than in the rabbit, though the maximum inhibition (specific activity) was almost equivalent (~70%) in both species. Nevertheless, a significant contractile elevating effect of SEA0400 was only found at a concentration of 1.0 μM when the NCX block was almost maximal in both species. This proposes that the slight difference in the affinity of the drug to the rat or rabbit NCX is not the main reason for the species dependent contractile force difference in the present study.

4.4.2. The effect of NCX on contractility is determined by species-dependent and pathological factors

1.0 μM SEA0400 was found to increase the contractile force, the cell shortening and the Ca^{2+} transient amplitude in an isolated ventricular tissue preparation of the mouse, which possesses a very short action potential duration [91]. In a recent study with isolated rat myocytes, the amplitude of the intracellular Ca^{2+} transient and cell shortening was significantly increased by SEA0400 in field stimulated and voltage clamped myocytes [33]. These isolated rat cell results are consistent with our whole rat heart contractile force results. In contrast, in the guinea-pig myocardium, which exhibits a longer action potential duration, 1.0 μM SEA0400 increased the contractile force by only 5% [92]. SEA0400 at a concentration of 1.0 μM significantly decreased the ouabain-induced inotropy when the reverse mode of NCX operation was favoured due to the high intracellular Na^+ (Na_i) level in the guinea-pig myocardium [92]. 1.0 μM SEA0400 did not affect the contractile force or the contractile force decay under normal conditions in guinea-pig myocardium, but it significantly enhanced the recovery of the contractile force in ischaemia/reperfusion [93]. This is supported by our findings in the healthy rabbit heart, which has a longer action potential duration, since 1.0 μM

SEA0400 did not elevate the contractile force significantly. In myocardial stunning in anaesthetized dogs, SEA0400 had no direct effect on contractility [94]. However, 1.0 μM SEA0400 significantly improved the recovery of the post-ischaemic left ventricle pressure in the rabbit heart [95] and in the rat heart [73]. This SEA0400 concentration likewise increased the contraction in rat hearts with a short action potential duration in our study, though under physiological conditions.

The above-mentioned findings emphasize the importance of different species-dependent and pathological factors of NCX functioning. The results imply that, under physiological conditions, NCX inhibition increases the contractility only in species which possess a short action potential duration; under pathological conditions NCX inhibition may increase the contractile force even in species with a longer action potential duration.

4.4.3. Species-dependent relation between the NCX function and contractility: possible mechanisms

The direction of operation of the NCX depends on I. the intracellular and the extracellular Ca^{2+} and Na^{+} concentrations, II. the prevailing phase and the shape of the action potential, and III. the pH [96]. Physiologically, the intracellular pH does not differ considerably in the rat heart and the rabbit heart. In contrast, the rabbit and the rat differ in some functional properties (e.g. AP and intracellular Na^{+}) as concerns the operation of the NCX [4]. The action potential duration of the rat (30-60 ms) is much shorter than that of the rabbit (150-200 ms) [97]. The actual membrane potential (E_m) and the reversal potential of the NCX ($E_{Na/Ca}$) define the direction of operation of the NCX: $E_{Na/Ca}$ depends on the Na_i which is higher in the rat (12.7-16.0 mM) than in the rabbit (7.2-9.0 mM) [4]. During depolarization, E_m is more positive than $E_{Na/Ca}$, which favours the reverse mode (Ca^{2+} influx). When E_m becomes more negative, the NCX operates in the forward mode (Ca^{2+}efflux). Because of the shorter action potential and higher $Na_{i,}$ in rats the NCX operates mainly in forward mode, even in systole [4] and the functional role of Ca^{2+} extrusion through the forward mode of the NCX is larger in species with a shorter action potential duration [92]. The longer action potential duration of the rabbit allows the NCX to operate in the reverse mode for a longer duration as compared with that in the rat. Accordingly, inhibition of the NCX operation in the settings of a short action potential in the mouse or the rat mainly affects the Ca^{2+} efflux (forward mode), which may result in intracellular Ca^{2+} accumulation on a beat-to-beat basis in the cardiac cycle under physiological conditions, thereby leading to an increased contractile force. In contrast, contractility fails to increase significantly in species with the settings of a

longer action potential (e.g. guinea-pig, rabbit and dog). Our rat and rabbit heart contractility results support this hypothesis. Despa and Bers found that NCX blocking Ni^{2+} reduced resting Na^{+} influx by almost 40 % in rat [98], which may underline the important role of the NCX in the Ca^{2+} extrusion in rats.

The inhibitory effect of SEA0400 can be influenced by the level of Na_i. The inhibitory potency of SEA0400 was reported to decrease 10-fold in response to a 4-fold decrease in Na_i [99]. This may suggest that the interspecies difference in the contractility response to the NCX inhibition by SEA0400 was due to the different Na_i levels. However, NCX inhibition were almost identical in the rabbit and the rat myocytes, when the highest concentration of the SEA0400 was used, which increased the contractile force significantly in rats. Thus, clarification of the exact mechanism of the observed interspecies difference in the contractility response to NCX inhibition needs further investigations. Table 11 summarizes the species dependent differences between the rabbit and rat influence Ca^{2+} handling [4].

	Rabbit	**Rat**
1. Sarcolemmal fraction involved in SR junctions (%)	4 of surface and 20 of T-tubule	7 of surface and 44 of T-tubule
2. I_{CaL} channel activation and inactivation	more positive E_m activation, more negative E_m inactivation	more negative E_m activation, more positive E_m inactivation
3. Ca(2+) influx in 200ms and Ca(2+) influx in the AP waveform	smaller, higher 21	higher, smaller 14
4. Role of NCX in Ca(2+) removal during relaxation (%)	30	8
5. Ca(2+) removal of (2+)-ATPase in the absence of SR Ca(2+)	6	13
6. NCX dependent Ca(2+) decline of caffeine induced contractures	much higher	smaller
7. Role of reverse NCX in $[Na]_o$ withdrawal	smaller	higher
8. Rest decay of SR Ca(2+) during caffein contracture	loses Ca(2+) continuously	stable
9. Effect of caffeine and ryanodine on steady state twitch force (%)	90, 75	33, 12
10. Ca(2+) transport rate (Vmax) SR and NCX (μM/l cytosol/sec)	82, 46	207, 27
11. Fraction of activator Ca(2+) from Ica and SR Ca release (%)	23, 77	8, 92
12. Spontaneous SR Ca(2+) release	often	only shows at high $[Ca]_o$ or with Na pump inhibition
13. $[Na]_i$ (mM)	7.2-9	12.7-16
14. APD_{90} duration (ms)	150-200	30-60
15. AP shape		

Table 11. Species dependent differences between the rabbit and rat influence Ca^{2+} handling.

4.4.4. Contractility and NCX protein expression

The NCX protein expression may increase under certain pathological conditions, such as heart failure or myocardial hypertrophy [23]. The function and protein expression of the NCX decreases during the development of the mouse [100]. The overexpression of the NCX in genetically manipulated feline myocytes causes a decline in contractility [21]. Similarly, overexpression of the NCX in rabbit ventricular myocytes gave rise to a more pronounced decay of the contraction and a failure to increase the extent of shortening for the increased frequency of stimulation, which was in contrast with the findings obtained in the NCX-overexpressing transgenic mouse [101]. The overexpression of NCX protein by somatic gene transfer in rat myocytes enhanced both intracellular systolic Ca^{2+} and contraction amplitude at low stimulation rates (0.25Hz), while it reduced cell shortening at higher stimulation frequencies (>2Hz) [22]. The NCX protein expressions levels have not been compared between different species under physiological conditions. In the present study, the NCX protein densities in the rat and the rabbit were compared to ascertain whether the different contractility results were attributable to different levels of NCX protein expression. As the NCX is widely distributed over the cell surface [102], immunohistochemistry was performed in isolated myocytes from rat and rabbit hearts. The fluorescence density did not differ in the two species, though this does not exclude the existence of different NCX activities despite the similar protein expressions. A decreased level of phosphorylation of the NCX could lead to a net reduction of NCX activity in spite of an increased expression [23]. Phosphorylation and the activity of the NCX were not examined in this study. However, the different effects of NCX inhibition on contractility in rat and rabbit hearts cannot be attributed to different levels of NCX expression in this work.

5. CONCLUSIONS

Our *'in vitro* α_1-adrenoceptor stimulation and stretch study' indicated that neither a sustained load-induced constant high level of left ventricular stretch nor α_1-adrenoceptor stimulation nor their co-application, promote the generation of TdP in the setting of prolonged repolarization in isolated, Langendorff-perfused rabbit hearts. The roles of intracardiac α_1-adrenoceptor stimulation and a sustained load-induced left ventricular stretch in the provocation of TdP in the rabbit are therefore questionable. In contrast, our results suggest the importance of extracardiac α_1-adrenoceptor stimulation in TdP development in the *in vivo* rabbit heart.

The ‘in vivo dofetilide study’ reported that dofetilide-induced TdP was not determined by the baseline haemodynamics, repolarization properties, autonomic status, blood gases or serum ion concentrations. However, those animals that subsequently experienced TdP had more responsive baroreflex during phenylephrine infusion before dofetilide administration, which implies that vagal nerve activity might contribute to TdP genesis. We also cannot exclude that sympathetic activation assisted dofetilide to evoke TdP in our study. When measured in sinus rhythm, none of the repolarization-related parameters forecasted dofetilide-induced TdP at any stage of the experiment. However, when arrhythmic activity was involved in the measurement of the beat-to-beat variability of the RR and QT interval, the ‘real’ variability of the RR and QT intervals predicted subsequent TdP occurrence. The progressive increase in the ‘real’ variability of the RR and QT interval implies that irregularity of the ‘preceding’ non-complex ventricular arrhythmias and the electrical instability rose steeply during dofetilide administration, which may be a prerequisite for the development of complex, re-entrant arrhythmias e.g. TdP in the model.

The ‘NCX arrhythmia study’ revealed that NCX neither contributed to the initiation nor assisted the maintenance of dofetilide-induced TdP in the applied in vitro experimental model, in which inhibition of the I_{Na} and I_{CaL} currents successfully antagonized the genesis of this arrhythmia. Neither the prolongation of the QTc interval nor the elevation of the beat-to-beat variability of the QT interval correlated to the occurrence of dofetilide-induced TdP in this model. AV ablation resulted in a chaotic idioventricular rhythm, which might make the hearts more sensitive to the proarrhythmic activity of dofetilide in the applied isolated rabbit heart model.

Our ‘NCX contractility study’ has provided evidence that the selective inhibition of the NCX with SEA0400 increases the contractility in a concentration-dependent manner in the isolated rat heart with a short action potential duration and a higher intracellular Na^+ concentration, but it does not exert an appreciable influence on the contractile force in the isolated rabbit heart with a longer action potential duration and a lower intracellular Na^+ concentration. These data reveal important functional interspecies differences in contractility as a result of NCX inhibition. Since rabbit action potential resembles more the human action potential than that of rat, the NCX inhibition probably does not influence the contractile force of human heart under physiological circumstances. However, the effect of NCX inhibition on cardiac contractility under pathological conditions, e.g. heart failure, needs further investigations.

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