

**Pharmacological modulation of the cardiac Na⁺/Ca²⁺ exchanger: role in
the study of Ca²⁺ handling and possible therapeutic applications**

PhD Thesis

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2007

List of publications related to the subject of the Thesis

Full length papers

- I. Károly Acsai, Attila Kun, Attila S Farkas, Ferenc Fülöp, Norbert Nagy, Marianna Balázs, Norbert Szentandrassy, Péter P Nánási, Julius Gy Papp, András Varró, András Tóth: Effect of partial blockade of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger on Ca^{2+} handling in isolated rat ventricular myocytes.
European Journal of Pharmacology, In press, 2007.
Impact factor (2006): 2.522
- II. Péter Birinyi, Károly Acsai, Tamás Bányász, András Tóth, Balázs Horváth, László Virág, Norbert Szentandrassy, János Magyar, András Varró, Ferenc Fülöp, Péter P Nánási: Effects of SEA0400 and KB-R7943 on $\text{Na}^+/\text{Ca}^{2+}$ exchange current and L-type Ca^{2+} current in canine ventricular cardiomyocytes.
Naunyn-Schmiedeberg's Archives of Pharmacology, 372, 63-70, 2005.
Impact factor (2005): 2.098
- III. Zsolt A Nagy, László Virág, András Tóth, Péter Biliczki, Károly Acsai, Tamás Bányász, Péter P Nánási, Julius Gy Papp, András Varró: Selective inhibition of sodium-calcium exchanger by SEA0400-0400 decreases early and delayed afterdepolarization in canine heart.
British Journal of Pharmacology, 143, 827-831, 2004.
Impact factor (2004): 3.325

Published abstracts

- IV. András Tóth, Károly Acsai, Attila Kun, László Virág, Julius Gy Papp, András Varró: The effect of partial $\text{Na}^+/\text{Ca}^{2+}$ -exchanger blockage on Ca^{2+} -handling in isolated rat cardiomyocytes.
Journal of Molecular and Cellular Cardiology, 39, 185-185, 2005.
- V. Péter Biliczki, Zsolt Nagy, László Virág, Károly Acsai, Julius Gy Papp, András Varró: Effect of a specific sodium-calcium exchanger blocker Sea0400 on the ventricular action potential and triggered activity in dog ventricular muscle and Purkinje fiber.
European Heart Journal, 25, 479-479 Suppl S, 2004.

Introduction

Role of Na⁺/Ca²⁺ exchanger in the cardiac myocytes

The Na⁺/Ca²⁺ exchange is a countertransport mechanism located in the cell membrane of almost every mammalian cell type. In heart muscle it was identified about forty years ago by measuring the dependence of Ca²⁺ efflux from cardiac muscle on ionic composition. Later it was determined that it exchanges 3 Na⁺ for 1 Ca²⁺, which implies that its operation is electrogenic, i.e. results in electrical current flowing across the membrane. The direction of this current corresponds to the mode of operation of the Na⁺/Ca²⁺ exchanger. In forward mode operation (Ca²⁺ efflux), an inward depolarizing current flows into the cell, while the reverse mode (Ca²⁺ influx) results in a concomitant outward current. Resulting partly from its electrogenic property, activity of the Na⁺/Ca²⁺ exchanger has a strong dependence on the instantaneous value of the membrane potential. Due to this and to the strong dependence of the Na⁺/Ca²⁺ exchanger activity on the electrochemical gradients of the transported ions, operation of the Na⁺/Ca²⁺ exchanger follows a complicated time course during the cardiac cycle. In addition, besides influencing the thermodynamic driving forces, the intracellular Na⁺ and Ca²⁺ also regulate the Na⁺/Ca²⁺ exchanger in an allosteric manner, making the understanding of its in situ regulation and activity exceptionally difficult. From structural point of view, the Na⁺/Ca²⁺ exchanger protein is considered to consist of nine transmembrane segments that form the transporter region, and a large intracellular loop involved in the intrinsic regulation of the Na⁺/Ca²⁺ exchanger by Na⁺ and Ca²⁺.

In cardiac myocytes, function of the Na⁺/Ca²⁺ exchanger belongs to the most important mechanisms of the Ca²⁺ homeostasis, and displays a major contribution to the regulation of the Ca²⁺ level during the cardiac excitation-contraction coupling. The major source of Ca²⁺ triggering the Ca²⁺ cycle is the L-type Ca²⁺ current which flows into the cell at the beginning of the action potential. In this phase of the action potential, the Na⁺/Ca²⁺ exchanger can contribute to the Ca²⁺ influx (reverse mode operation) into the cell since the membrane potential is positive and the intracellular Ca²⁺ level is low. However, when the intracellular Ca²⁺ level increases at the beginning of the Ca²⁺ transient due to the Ca²⁺-induced release of Ca²⁺ from the sarcoplasmic reticulum, the Na⁺/Ca²⁺ exchanger turns into its forward mode operation, thereby contributing to the extrusion of Ca²⁺ from the cell. Relaxation of the Ca²⁺ transient is a result of the activities of three competing mechanisms. 70-90 % of the cytoplasmic Ca²⁺ is sequestered back into the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca²⁺-ATPase, 7-30 % is extruded from the cell by the Na⁺/Ca²⁺ exchanger, and only a small amount of Ca²⁺ is extruded by the slow Ca²⁺-transport systems, such as the sarcolemmal Ca²⁺-ATPase and the mitochondrial Ca²⁺-transport. The Ca²⁺ balance of the cell requires that in steady state equilibrium, the beat-to-beat Ca²⁺ influx and efflux must be equal in the wide range of physiological conditions to avoid Ca²⁺ loss or Ca²⁺ overload in the cell, which means that the main transmembrane Ca²⁺ fluxes in both directions need to be finely regulated. The general outline of the function of Na⁺/Ca²⁺ exchanger can show considerable variations depending on the experimental circumstances and the species under examination. For example, there is a continuous debate about the involvement of the reverse mode Na⁺/Ca²⁺ exchanger in the initiation of Ca²⁺ induced Ca²⁺ release and thus the systolic Ca²⁺ transient and cell contraction. Similarly, although the crucial role of the forward mode Na⁺/Ca²⁺ exchanger in extrusion of Ca²⁺ from the cell is generally accepted, its relative contribution to the total extruded Ca²⁺ can show significant variations depending on species and physiological/ pathological situations. Furthermore, there are emerging hypotheses

regarding some unexplored roles of the Na⁺/Ca²⁺ exchanger, such as its suggested function in buffering the cytoplasmic Ca²⁺ movements. Due to its crucial importance in the Ca²⁺ handling and the unresolved questions regarding its regulation, the role of Na⁺/Ca²⁺ exchanger in the cardiac cycle under physiological and pathological conditions has been extensively studied in the recent years.

Pharmacology of the cardiac Na⁺/Ca²⁺ exchanger

An important way to examine the role of a transport system in physiological or pathological situations is to block the transporter with a selective inhibitor. Until recently, however, this possibility was hampered in case of Na⁺/Ca²⁺ exchanger because of the lack of potent and highly specific inhibitors. Therefore, although there are several pharmacological agents inhibiting the Na⁺/Ca²⁺ exchanger, the interpretation of results obtained using these compounds is complicated by the concomitant effects on other transport systems or ionic channels. For example, amiloride analogs have been used to study Ca²⁺ homeostasis in cardiac preparations, but these agents have also been shown to block the voltage gated Ca²⁺ channels, making the interpretation of their effects regarding the role of Na⁺/Ca²⁺ exchanger in Ca²⁺ handling rather difficult. Other pharmacological agents such as bepridil, amiodarone or tetracaine are also able to inhibit the Na⁺/Ca²⁺ exchanger, but again, these molecules are nonselective and the concentrations at which they inhibit the Na⁺/Ca²⁺ exchanger are often even higher than the concentrations at which they exert their primary actions. The exchanger inhibitory peptide (XIP), which resembles a calmodulin binding domain, has also been used to block the Na⁺/Ca²⁺ exchanger current, but its application is limited in physiological experiments because the peptide must be applied intracellularly and it also inhibits other Ca²⁺ transport systems. Several trivalent and divalent cations are also capable to inhibit the Na⁺/Ca²⁺ exchanger, among which Ni²⁺ has been used extensively to identify the Na⁺/Ca²⁺ exchanger current in electrophysiological experiments. The main advantage of using Ni²⁺ is that it blocks Na⁺/Ca²⁺ exchanger totally in a reversible manner. In general, these nonselective molecules and ions can only be used in subcellular systems or in experiments in which the Na⁺/Ca²⁺ exchanger is isolated by means of applying other blockers and ionic substitutions.

Therefore, the recently developed potent and selective Na⁺/Ca²⁺ exchanger inhibitors present a great advance in the field of the Na⁺/Ca²⁺ exchanger research, as by using these molecules the role of the Na⁺/Ca²⁺ exchanger can be investigated in intact cells or tissues under physiological circumstances. The two most selective and widely used Na⁺/Ca²⁺ exchanger inhibitors in the literature are the aniline derivative SEA0400 (2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxy-aniline) and the isothiourea derivative KB-R7943 (2-[2-[4-94-nitrobenzyloxy]phenyl]-ethyl]isothiourea). When examining the role of the Na⁺/Ca²⁺ exchanger in physiological as well as pathological circumstances by using pharmacological tools, it is important to know the selectivity, potency and possible direction dependence of the inhibitors. Therefore, the first aim of the present work was to compare the pharmacological profile of the two recently introduced Na⁺/Ca²⁺ exchanger inhibitor, KB-R7943 and SEA0400.

An early study investigating the pharmacological effect of KB-R7943 concluded that this compound affects the Na⁺/Ca²⁺ exchanger in a Ca²⁺-dependent manner under certain experimental circumstances, suggesting that an interaction might exist between the drug and the intrinsic regulatory mechanisms of the Na⁺/Ca²⁺ exchanger. Similar interaction has been suggested to be involved in the mechanism of action of SEA0400, as its blocking effect was found to be dependent on the intracellular Na⁺ concentration, and related inversely to the intracellular Ca²⁺ concentration. However, these experiments were carried out in an expressed

system using non-physiological ionic concentrations to measure the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current. Therefore, our second aim in this work was to investigate the possible interaction between the intracellular Ca^{2+} concentration and the inhibitory effect of SEA0400 in adult cardiac myocytes using physiological ionic concentrations.

Therapeutic potential of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors

In the recent years, several studies indicated that inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can be beneficial in experimental models of cardiac disorders. Most of these studies examined the involvement of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the ischemic damage of the myocardium. Based on the thermodynamics of the operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, intracellular accumulation of Na^+ in ischemic/reperfused myocardium favors the reverse mode operation of the exchanger, which may contribute to the intracellular accumulation of Ca^{2+} leading to cell damage and death. Therefore, it can be speculated that pharmacological inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may display cardioprotective effects under such circumstances. In accordance with this hypothesis, it has been reported that SEA0400 and KB-R7943 reduced infarct size in rat and rabbit hearts after ischemia/reperfusion. The beneficial effects exerted by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors in ischemia/reperfusion were also demonstrated in large animal models such as dog and pig. The above and other studies also examined the proposed mechanisms of the observed protective effects of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors in such circumstances. Indeed, it has been verified that the most likely mechanism of action was the prevention of the excessive Ca^{2+} overload by inhibiting the reverse mode operation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

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

Most of the above studies investigated end point parameters related to pathological consequences of myocardial Ca^{2+} overload, like contractile dysfunction, infarct size, or incidence of arrhythmias. However, although it is closely related to the potential therapeutic uses of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors, relatively few works investigated directly the effects of the reduced $\text{Na}^+/\text{Ca}^{2+}$ exchanger function on the Ca^{2+} homeostasis itself. KB-R7943, XIP or non-pharmacological tools were used in these studies, but to the our best knowledge, there was no such study using SEA0400. Therefore, the next aim in this work was to directly study how partial inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchanger by the selective pharmacological agent SEA0400 influences the elements of Ca^{2+} handling in cardiac cells from rat and dog. Finally, the positive inotropic effect observed in rat cardiac myocytes was investigated further regarding the possible autoregulative changes in the Ca^{2+} homeostasis that can be expected to occur after any disturbance in the steady state balance of the cellular Ca^{2+} fluxes.

Major experimental goals

1. Comparison of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger blocking potency and its possible direction dependence of SEA0400 and KB-R7943 in whole cell patch clamp experiments.
2. Investigation of the possible relationship between the intracellular Ca^{2+} concentration and the inhibitory action of the SEA0400 in whole cell patch clamp experiments.
3. Effect of $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibition by SEA0400 on Ca^{2+} transients in intact field stimulated rat and dog cardiac myocytes.
4. Further analysis of the effect of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibition by SEA0400 on Ca^{2+} cycling in rat cardiac myocytes by applying the perforated patch clamp technique.

Materials and methods

Animal care

The studies were conducted in accordance with the standards of the European Community guidelines on the care and use of laboratory animals and the protocols had been approved by the Ethical Committee for Protection of Animals in Research of the University of Szeged, Szeged, Hungary.

Measurement of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger current ($I_{\text{Na/Ca}}$) in isolated dog ventricular cells and estimation of the blocking potency of SEA0400 and KB-R7943

Single canine cardiac myocytes were obtained by enzymatic dissociation of the hearts. $\text{Na}^+/\text{Ca}^{2+}$ exchanger current ($I_{\text{Na/Ca}}$) was recorded in whole cell patch clamp experiments as a Ni^{2+} sensitive current. This method is based on the observation that Ni^{2+} blocks the $I_{\text{Na/Ca}}$ completely. After establishing the whole-cell configuration in normal Tyrode solution, the cell was superfused with a special K^+ -free bath solution supplemented with 20 μM ouabain, 1 μM nisoldipine and 50 μM lidocaine in order to block Na^+ , K^+ , Ca^{2+} and Na^+/K^+ pump currents. $I_{\text{Na/Ca}}$ was recorded using ramp pulses (having a velocity of 100 mV/s) delivered at a rate of 0.05 Hz. Outward and inward $I_{\text{Na/Ca}}$ were determined during the descending limb of the ramp at +40 and -80 mV. After taking the control record in K^+ -free solution, the cell was superfused with the given concentration of SEA0400 or KB-R7943, and finally 10 mM NiCl_2 was added in order to fully block $I_{\text{Na/Ca}}$. Thus, total $I_{\text{Na/Ca}}$ was determined at both membrane potentials as a Ni^{2+} -sensitive current.

Dependence of the inhibitory action of SEA0400 on the intracellular Ca^{2+} concentration

Ca^{2+} dependence of the exchanger inhibiting effect of SEA0400 was investigated in dog myocytes using the protocol described above, except that the free Ca^{2+} concentration of the pipette solution was buffered to different levels by adding 5 or 15 mM Ca^{2+} to 20 mM EGTA, and 5.3 or 14 mM Ca^{2+} to 20 mM BAPTA. Calculated free Ca^{2+} concentrations were 55 and 500 nM with EGTA, and 140 and 1000 nM in case of BAPTA, respectively.

Measurement of intracellular Ca^{2+} concentration and cell shortening in field stimulated rat and dog ventricular cells

Single canine and rat cardiac myocytes were obtained by enzymatic dissociation of the hearts. Changes in intracellular free Ca^{2+} concentration were assessed by the ratiometric fluorescence technique using Fura-2-AM. Cells were superfused in the experimental chamber with normal Tyrode solution at 35 °C and stimulated using an electronic stimulator at 1 or 4 Hz via a pair of platinum electrodes. Changes in intracellular free Ca^{2+} levels were approximated by the ratio of the fluorescence intensity obtained at 360 and 380 nm excitation. Cell shortening was recorded using a video edge detector system. Concentrations of SEA0400 applied in the superfusion solution were 0.3 μ M in the experiments on rat myocytes, and 0.1, 0.3 and 1 μ M in case of dog myocytes. In separate experiments, activity of the Na^+/Ca^{2+} exchanger was modulated in dog myocytes by decreasing the extracellular Na^+ concentration from 144 to 70 mM (replaced with choline-chloride), and changes in the Ca^{2+} transients were recorded as described above.

Recording the caffeine induced Ca^{2+} transients in patch clamped dog cardiac myocytes

Cells loaded with Fura-2-AM were superfused in the experimental chamber with normal Tyrode solution at room temperature. After establishing the whole cell configuration, conditioning depolarization pulses were applied to the cell at a frequency of 0.5 Hz in order to standardize the sarcoplasmic reticulum Ca^{2+} content. Then the holding potential was set to -80 mV to prevent any disturbances resulting from the changes of the membrane potential and with a standardized delay, total release of the sarcoplasmic reticulum Ca^{2+} content was induced by rapid application of 10 mM caffeine via a side arm of the cell chamber. The caffeine induced Ca^{2+} transients and total membrane current were recorded in the absence and presence of 10 mM Ni^{2+} in order to investigate the relative involvement of the Na^+/Ca^{2+} exchanger in the relaxation of the Ca^{2+} transients.

Recording the L-type Ca^{2+} current, intracellular Ca^{2+} transient, and cell shortening in patch clamped rat cardiac myocytes

Perforated cell membrane configuration of the patch clamp technique was used to record the L-type Ca^{2+} current in parallel with the recording of intracellular Ca^{2+} transient. Cells loaded with Fura-2-AM were superfused at 35 °C with normal Tyrode solution containing 5 mM of 4-aminopyridine and 0.15 μ M of $BaCl_2$ to suppress K^+ currents. Pipette solution was supplemented with 220 μ g/ml amphotericin B in order to develop a reasonably good electrical access to cell interior. L-type Ca^{2+} currents and the intracellular Ca^{2+} transients were evoked by a composite voltage command. Effect of SEA0400 on voltage-current characteristic of the L-type Ca^{2+} current was measured in whole cell patch clamp configuration by applying square pulse voltage protocol.

Drugs

All chemicals, except SEA0400 were purchased from Sigma-Aldrich. SEA0400 was synthesized at the Department of Pharmacological Chemistry (University of Szeged, Szeged, Hungary). SEA0400 was dissolved in dimethyl sulfoxide (vehicle) and its final concentrations were 0.1, 0.3 or 1 μ M when diluted in Tyrode solution.

Results

Comparison of the estimated EC_{50} values of SEA0400 and KB-R7943

In dog cardiac myocytes SEA0400 and KB-R7943 suppressed both inward and outward $I_{Na/Ca}$ in a concentration-dependent manner. The most striking difference between the effects of SEA0400 and KB-R7943 was the difference in their affinity: the estimated EC_{50} value was lower for SEA0400 (0.11 μ M for both direction) than KB-R7943 (4.74 μ M for outward and 3.35 μ M for inward current) by approximately 1.5 orders of magnitude. No significant difference was seen in the SEA0400-induced suppression of inward and outward $I_{Na/Ca}$. In contrast, KB-R7943 suppressed outward $I_{Na/Ca}$ more effectively than inward $I_{Na/Ca}$ at concentrations higher than 3 μ M. The maximal blockade of inward $I_{Na/Ca}$ produced by the highest concentration of KB-R7943 (50 μ M) was only 44.6 \pm 9.1%. The second difference seen between the effects of the two drugs was in their Hill coefficients: a value of close to unity was obtained for SEA0400, whereas it was around 2 in the case of KB-R7943.

Ca^{2+} dependence of the Na^+/Ca^{2+} exchanger blockade by SEA0400 in patch clamped dog cardiac myocytes

Experimental groups were constructed according to the concentrations of the free Ca^{2+} used in the patch pipettes. With the increasing concentrations of the free Ca^{2+} in the pipette solution, SEA0400 exhibited a significant decreasing tendency in its blocking effect on both outward and inward $I_{Na/Ca}$, resulting in significant differences between the groups of lower and higher Ca^{2+} levels (outward current: 58.4 \pm 8.3 % in 55 nM Ca^{2+} group versus 39.1 \pm 6.7 % in the 500 nM Ca^{2+} group, $P < 0.05$; inward current 78.2 \pm 9.3 % in 55 nM Ca^{2+} group versus 42.7 \pm 4.7 % in the 500 nM Ca^{2+} group, $P < 0.05$). Regarding the outward current, the percent inhibition showed a monotone decrease, but the blocking values observed in the 500 and 1000 nM Ca^{2+} level groups did not differ from each other neither in case of the outward nor in the inward current. This may suggest that the critical Ca^{2+} level in the inverse Ca^{2+} -dependent Na^+/Ca^{2+} exchange blocking phenomenon may be in the lower range of the physiological Ca^{2+} levels.

Effect of SEA0400 on the intracellular Ca^{2+} transient and cell shortening in field stimulated rat cardiac myocytes

Rat cardiac myocytes were stimulated at a constant frequency of 4 Hz through a pair of platinum electrodes. Vehicle alone failed to affect the intracellular Ca^{2+} transient and cell shortening. In contrast, administration of SEA0400 at a concentration of 0.3 μ M resulted in an increased amplitude of both intracellular Ca^{2+} transient and cell shortening. In some experiments a moderate elevation in diastolic Ca^{2+} and baseline cell length was observed after SEA0400. The SEA0400-induced increase in the amplitude of Ca^{2+} transient (31.6 \pm 8.8 % in SEA0400, $n = 6$ versus 8.5 \pm 3.8 % in vehicle, $n = 7$, $P < 0.05$) and in cell shortening (45.5 \pm 14.9 % in SEA0400 versus 2.0 \pm 15.2 % in vehicle, $P < 0.05$) were statistically significant, while elevation of the diastolic Ca^{2+} and baseline cell length was moderate and not significant statistically (6.1 \pm 3.3 % in SEA0400 versus 0.9 \pm 1.4 % in vehicle, $P = 0.13$, for diastolic Ca^{2+} ; and 5.5 \pm 2.9 % in SEA0400 versus 3.0 \pm 0.8 % in vehicle, $P = 0.22$, for baseline cell length).

However, as the Na^+ -dependent inactivation process was found to be dependent not only on Na^+ but also on the regulatory Ca^{2+} , i.e. increasing the concentration of the Ca^{2+} at the cytoplasmic surface decreased the rate and maximal extent of the Na^+ -dependent inactivation, it can be expected that the intracellular Ca^{2+} can also interact indirectly with the effect of SEA0400. Indeed, with increasing the cytoplasmic free Ca^{2+} from 1 to 10 μM , an inverse Ca^{2+} -dependent inhibitory effect produced by SEA0400 can be demonstrated. This finding may be especially important regarding the action of SEA0400 on intact cardiac muscle, since the changes in the intracellular Ca^{2+} levels during the cardiac cycle is more pronounced than that of the intracellular Na^+ .

In connection with this issue we have shown in our work that the inverse Ca^{2+} -dependent inhibitory effect of SEA0400 can also be demonstrated in whole cell patch clamp experiment with ionic concentrations more closely resembling the physiological values. At the same time our results indicate that the inverse Ca^{2+} -dependent inhibitory effect of SEA0400 occurs not only in expressed systems but also in adult mammalian cardiac myocytes. Furthermore, we demonstrated that this phenomenon applies not only to the reverse but also to the forward mode operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

The highest concentration of the intracellular free Ca^{2+} applied in our experiments was 1 μM , which corresponds to the peak value of the intracellular Ca^{2+} transient measured by fluorescent Ca^{2+} indicators. At this Ca^{2+} level the observed inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger by 1 μM SEA0400 was about 50 % of its maximal inhibitory effect, which is still a considerable value. However, during the Ca^{2+} cycle, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can sense significantly higher intracellular Ca^{2+} concentrations, especially at the subsarcolemmal space in the t-tubules, where the Ca^{2+} is released from the sarcoplasmic reticulum release channels. Furthermore, at the regions of the t-tubules, the functional density of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is about 3-4 fold higher than in external sarcolemma. Therefore, in cardiac myocytes with intact Ca^{2+} handling the actual Ca^{2+} -dependent decrease in the effect of SEA0400 can be more pronounced than it can be predicted from whole cell patch clamp experiments. The phenomenon of the inverse Ca^{2+} -dependent inhibitory effect also implies that in intact cardiac myocytes the momentary inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger produced by a given concentration of SEA0400 may change dynamically during the cardiac cycle, from its maximal value during diastole when the level of intracellular Ca^{2+} is low, to a pronouncedly blunted level when the intracellular free Ca^{2+} increases due to the Ca^{2+} -induced Ca^{2+} release. However, as we measured the exchanger current in steady state conditions only, this possibility requires further support from experiments that allow dynamic measurement of this current.

Practical consequences of this inverse Ca^{2+} -dependent $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibition is unknown currently, but it may limit the effectivity of the actual inhibition produced by SEA0400 in intact cardiac myocytes, which means that the inhibitory potency of the SEA0400 can be overestimated using steady state protocols in whole cell patch clamp experiments. From the point of view of possible therapeutic uses of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors, the inverse Ca^{2+} -dependent exchanger inhibition can be an advantageous property, as it may limit the excessive blocking of the forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchanger in intact cardiac myocytes, by which the dangerous Ca^{2+} overload can be avoided.

Positive inotropic effect of SEA0400 in field stimulated rat cardiac myocytes

When investigating the potential therapeutic uses of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors, it is important to explore their effect in the Ca^{2+} homeostasis, as changes occurring in the Ca^{2+} handling play a crucial role in the pathophysiology of most cardiac disorders. Therefore, we have investigated the effect of partial inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger on the Ca^{2+} transient, which is a central parameter in the Ca^{2+} cycling in intact cardiac myocytes. Theoretically, consequences of a partial $\text{Na}^+/\text{Ca}^{2+}$ exchanger blockade depends on the relative degree of inhibition of the reverse and forward mode operation of $\text{Na}^+/\text{Ca}^{2+}$ exchanger, as well as on the relative contribution of the two modes of $\text{Na}^+/\text{Ca}^{2+}$ exchanger operation to the Ca^{2+} balance of the cell. For example, it has been shown that KB-R7943, a compound that predominantly blocks the reverse mode operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and thereby decreases the Ca^{2+} influx via $\text{Na}^+/\text{Ca}^{2+}$ exchanger, failed to affect the amplitude of either Ca^{2+} transients or contractions in rat cardiac myocytes suggesting that in this species the contribution of reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchanger to the total Ca^{2+} influx is negligible. However, both parameters were significantly decreased by KB-R7943 in guinea pig myocytes, indicating that in this species the Ca^{2+} influx via the reverse mode $\text{Na}^+/\text{Ca}^{2+}$ exchanger plays a significant role in the initiation of the excitation contraction coupling. In contrast to KB-R7943, we and others have demonstrated that SEA0400 equally inhibits the forward and reverse mode operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in patch clamped myocytes. Based on these data, in rat cardiac myocytes accentuated consequences of the forward mode blockade of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger may be anticipated with SEA0400. Indeed, according to our results 0.3 μM SEA0400 significantly increased the amplitude of Ca^{2+} transients and the cell shortening in field stimulated rat cardiac myocytes suggesting that significant intracellular accumulation of Ca^{2+} occurs as a consequence of the inhibition of Ca^{2+} efflux mode of the exchanger.

In physiological conditions, the three main Ca^{2+} transport systems that compete for Ca^{2+} during the relaxation phase of the Ca^{2+} transient are the sarcoplasmic reticulum Ca^{2+} -ATPase, the forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the sarcolemmal Ca^{2+} -ATPase. It can be assumed that if one of these mechanisms is inhibited, the decrease in the capacity of the Ca^{2+} removal systems results in slowing of the Ca^{2+} extrusion process, that is, the time constant of the relaxation rate can increase. However, an important finding in our experiments is that in intact cardiac myocytes the time constant of the Ca^{2+} transient relaxation does not change after the partial inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, suggesting that the role of the exchanger is negligible in governing the relaxation. This possibility is supported by the finding that the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is found predominantly in the t-tubules of the sarcolemmal membrane, playing an important role in regulating the Ca^{2+} level near to the sarcoplasmic reticulum release channels during the Ca^{2+} -induced Ca^{2+} release, i.e. during the upstroke of the Ca^{2+} transient, and having a significantly less role during the relaxation phase of the Ca^{2+} transient. Therefore, if the $\text{Na}^+/\text{Ca}^{2+}$ exchanger is inhibited, the amplitude of the Ca^{2+} transient can reach a higher value due to the diminished Ca^{2+} efflux from the vicinity of the Ca^{2+} transients, but the relaxation of the Ca^{2+} transient, although begins at an elevated Ca^{2+} level, can proceed with the same (or even elevated) rate constant, which is determined mainly by the sarcoplasmic reticulum Ca^{2+} -ATPase.

An important finding in our experiments on rat cardiac myocytes was to show that partial inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchanger may enhance contractility without significant elevation of the diastolic Ca^{2+} level. Although SEA0400 tended to increase the diastolic Ca^{2+} level, this tendency remained within the range of experimental variability. However, it must

be kept in mind that when the Ca^{2+} extruding capacity of the cell is compromised due to partial inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, application of other agents influencing diastolic Ca^{2+} should be avoided in order to prevent the elevation of proarrhythmic risk, since an otherwise minor effect on diastolic Ca^{2+} can be potentiated by the reduced Ca^{2+} extruding capacity. The effect of $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibition on diastolic Ca^{2+} has particular importance, because in heart failure the diastolic Ca^{2+} is elevated, and in this case application of drugs further increasing diastolic Ca^{2+} are contraindicated. From this point of view our results obtained with SEA0400 are in line with those obtained in an other study using the exchange inhibitory peptide (XIP) applied intracellularly to study the effect of the reduced $\text{Na}^+/\text{Ca}^{2+}$ exchanger activity on Ca^{2+} handling in canine ventricular myocytes. In that study, an increased amplitude of the Ca^{2+} transient was observed without significant increase in diastolic Ca^{2+} concentration. This may be due to the Ca^{2+} -dependent stimulation of sarcoplasmic reticulum Ca^{2+} uptake, which is an important autoregulatory mechanism against cytoplasmic Ca^{2+} overload. It is reasonable to assume that such a phenomenon may also occur under our experimental conditions, contributing to the increased sequestration of Ca^{2+} into the sarcoplasmic reticulum, which results in the observed positive inotropic action.

SEA0400 fails to affect the intracellular Ca^{2+} transient in field stimulated dog cardiac myocytes

Interestingly, in the same experimental setting a similar increase in the amplitude of Ca^{2+} transients in dog myocytes was not observed after administration of SEA0400 at 0.3 and even 1 μM concentration. The reason for this discrepancy between the results obtained in rat and dog myocytes is unknown. As SEA0400 proved to be a potent inhibitor of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in dog myocytes in whole cell patch clamp experiments, the possibility that SEA0400 did not inhibit the $\text{Na}^+/\text{Ca}^{2+}$ exchanger isoform or splice variant that is expressed in dog myocytes can be excluded. However, since the EC_{50} values of SEA0400 are very similar for inhibition of both the reverse and forward mode operation of the $\text{Na}^+/\text{Ca}^{2+}$ exchange, it can be assumed that SEA0400 equally inhibits both the Ca^{2+} influx and efflux mediated by the exchanger during the cardiac Ca^{2+} cycle, and thus its net effect does not cause any significant change in the Ca^{2+} transient. This explanation implies, however, that in dog cardiac myocytes the amount of Ca^{2+} transported by the reverse and forward mode exchanger is similar, which is not likely, because the majority of the Ca^{2+} influx is mediated by the L-type Ca^{2+} current. Thus this possibility can be held only if the blocked part of the Ca^{2+} efflux via the forward mode exchanger is counterbalanced by other Ca^{2+} efflux systems, most likely by the sarcolemmal Ca^{2+} pump.

The contribution of the different Ca^{2+} efflux systems to the extrusion of Ca^{2+} from the cytoplasm varies considerably between different species. In dog cardiac myocytes, however, this issue is less investigated. The only study available in the literature showed that the time required to achieve half relaxation during caffeine exposure (i.e. when the sarcoplasmic reticulum Ca^{2+} uptake is inhibited) showed a 4.4-fold increase when the $\text{Na}^+/\text{Ca}^{2+}$ exchanger was totally inactivated by application of Na^+ and Ca^{2+} free solution, comparing to the value obtained when both the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the slow Ca^{2+} transport systems were active. In order to examine the possibility that in dog myocytes the sarcolemmal Ca^{2+} pump or other Ca^{2+} transport mechanisms can present a reserve system in the extrusion of Ca^{2+} from the cell we compared the relaxation rates of the caffeine induced Ca^{2+} transients in the presence and absence of fully active $\text{Na}^+/\text{Ca}^{2+}$ exchanger, i.e. before and after total inhibition of the exchanger by 10 mM Ni^{2+} . Relaxation of the Ca^{2+} transient proceeded more slowly after total inhibition of the Ca^{2+} removal via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, resulting in a 4.8-fold increase in

the half relaxation time. However, data from the literature show that in similar experimental setting using rat and rabbit myocytes, 8- and 10-fold increase can be seen in the relaxation time after inactivation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. As these values relate inversely to the Ca^{2+} extruding capacity that remains after inactivation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, these results indicate that in dog ventricular myocytes the Ca^{2+} removing systems other than the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can display higher capacity than in other species. Therefore, although this type of experiment does not give any specific information about the dynamic interaction occurring between the different Ca^{2+} removal systems during the intact Ca^{2+} cycle, we can speculate that the lack of the effect of SEA0400 on dog cardiac myocytes can be explained, at least in part, on the basis of a significant Ca^{2+} extruding capacity of the cell that can counterbalance the blocked part of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger.

To test further the hypothesis that the similar degree of inhibition exerted by SEA0400 on both operating direction of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can account for the lack of apparent effect on the Ca^{2+} handling in dog cardiac myocytes, we measured the Ca^{2+} transient in normal conditions and after decreasing the extracellular Na^+ level from its normal value of 144 mM to 70 mM. This intervention, in contrast to the effect of SEA0400, exerts an opposite effect on the two direction of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The lower extracellular Na^+ level decreases the forward mode operation while favours the reverse mode operation at any given intracellular concentrations of Na^+ and Ca^{2+} , which is due to a shift of the equilibrium potential of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to more positive values. Therefore, the Ca^{2+} efflux via the exchanger is inhibited partially as in the case of SEA0400, but, in contrast to SEA0400, the Ca^{2+} influx via the reverse mode exchanger increases, and theoretically the new equilibrium in the Ca^{2+} cycling develops with increased Ca^{2+} influx and elevated intracellular Ca^{2+} level. Indeed, we found a clear cut elevation in the amplitude of the Ca^{2+} transient after decreasing the extracellular Na^+ level. However, this was only a moderate increase, which can suggest that if only a limited increase can be achieved in the Ca^{2+} transient when both the Ca^{2+} influx and Ca^{2+} efflux mode are modulated in the direction of Ca^{2+} accumulation, then it is possible that in case of SEA0400 the effects of decreased Ca^{2+} influx and efflux cancel each other, resulting in negligible effect on the Ca^{2+} transient.

A different explanation for the lack of effect of SEA0400 on Ca^{2+} handling in dog cardiac myocytes involves the ionic dependence of the inhibitory effect of SEA0400. As we have discussed above the inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger produced by SEA0400 requires the presence of intracellular Na^+ , which allows the Na^+ -dependent inactivation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to develop and SEA0400 accelerates this process. From this point of view, the intracellular Na^+ has a permissive effect for the development of the inhibitory effect of SEA0400, and more pronounced consequences of the application of SEA0400 can be expected with higher intracellular Na^+ levels. Congruent with this property, we have found a clear cut effect on the Ca^{2+} transient in rat, in which species the cytoplasmic Na^+ concentration in the cardiac myocytes is considerably higher than in the myocytes from other species. This finding may have practical importance, as it is known that in heart failure the intracellular Na^+ concentration is elevated. Therefore, appearance of the positive inotropic action of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibition can be expected in case of diseased heart compared to healthy heart. Further studies are required to prove these speculations.

Whatever the background of the observed differing effect of SEA0400 in rat and dog myocytes, it is possible that this difference mainly results from the unique mechanism of action of SEA0400, as inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger by other means explicitly results in positive inotropic effect in dog myocytes as well. Therefore, we can conclude that pharmacological inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can present a new mechanism in the positive inotropic therapy of heart failure.

Further analysis of the changes in Ca^{2+} cycling caused by SEA0400 in rat ventricular myocytes

Although SEA0400, applied at a concentration of 0.3 μ M was shown to block at least 50% of Na^+Ca^{2+} exchanger activity, elevation of the intracellular Ca^{2+} transient observed in rat cardiac myocytes was not accompanied by signs of Ca^{2+} overload after the application of SEA0400 in the present study. It is likely, therefore, that according to the theory of autoregulation of Ca^{2+} handling in cardiac myocytes, a new steady state equilibrium in Ca^{2+} cycling has developed in the presence of SEA0400. In this case, Ca^{2+} influx had also to be decreased in order to achieve the new equilibrium between Ca^{2+} influx and efflux. To test this hypothesis we recorded the L-type Ca^{2+} current simultaneously with intracellular Ca^{2+} transients under perforated patch clamp conditions. The results showed that the amplitude of peak Ca^{2+} current actually decreased while its inactivation was accelerated after application of 0.3 μ M SEA0400. We also demonstrated that SEA0400 at this concentration failed to affect directly the L-type Ca^{2+} current in whole cell patch clamp experiments, corresponding to previous findings obtained in guinea pig and dog cardiac myocytes. Therefore, one may conclude that the reduced Ca^{2+} current is an indirect consequence of the decreased Ca^{2+} extrusion, and is likely associated with the increased amplitude of the intracellular Ca^{2+} transient. The main mechanism underlying this phenomenon may be the Ca^{2+} -dependent inactivation of the L-type Ca^{2+} channel, which provides a beat to beat basis for an automatic negative feedback mechanism playing a key role in the autoregulation of Ca^{2+} cycling. As the cytosolic Ca^{2+} increases due to inhibition of the forward mode operation of Na^+Ca^{2+} exchanger, the increased Ca^{2+} load may result in increased sequestration of Ca^{2+} into the sarcoplasmic reticulum, and thus providing more Ca^{2+} to be released. Congruent with this explanation the diastolic Ca^{2+} level slightly increased after application of SEA0400. Although this change in diastolic Ca^{2+} was moderate and remained below the level of statistical significance, it might be sufficient to gradually increase the Ca^{2+} content of the sarcoplasmic reticulum during the experimental period leading to increased Ca^{2+} released into the subsarcolemmal space, which in turn might enhance the inactivation of L-type Ca^{2+} channels. However, if the reduced Ca^{2+} entry is able to initiate an increased Ca^{2+} transient, the new equilibrium may also involve changes in the gain of the Ca^{2+} induced Ca^{2+} release under such conditions. Further studies are required to elucidate the exact nature of this new equilibrium.

It is worthy to mention that a study using adenovirus-mediated antisense oligonucleotide method to decrease the Na^+Ca^{2+} exchanger function came to different conclusions, as no significant change in the amplitude of Ca^{2+} transient was observed in rat myocytes after the diminished expression of the exchanger. This discrepancy between their and our results may be due to differences in experimental conditions (i.e. the methods used to reduce the activity of Na^+Ca^{2+} exchanger). The Na^+Ca^{2+} exchanger knockout induced by application of antisense oligonucleotids required 72 hours to develop, which allowed for adaptive changes in Ca^{2+} handling to occur, thus compensating for the compromised Na^+Ca^{2+} exchanger activity. Such adaptive changes are not likely to take place in our model, because of the acute application of the selective Na^+Ca^{2+} exchanger blocker. In addition, an opposite effect was seen concerning the L-type Ca^{2+} current as well, as they found no change in this current after reduction of Na^+Ca^{2+} exchanger activity. This difference can also be ascribed to differences in the experimental techniques, since they measured L-type Ca^{2+} current using the conventional patch clamp method having intracellular Ca^{2+} buffered to non-physiologically low level. In contrast, the perforated patch clamp technique applied in our experiments, allowed the changes in cytosolic Ca^{2+} to occur, and the observed reduction in the L-type Ca^{2+}

current was possibly a consequence of the dynamic autoregulative adaptation of Ca^{2+} cycling after the inhibition of the Na^+Ca^{2+} exchanger.

Therefore, we can conclude that the selective pharmacological inhibition of the Na^+Ca^{2+} exchanger as a research tool can be used in studying the Ca^{2+} homeostasis and its regulatory aspects in physiological experiments, and, at least in this type of experiments, its use may have advantages over the genetical methods. Moreover, regarding the possible therapeutic application of the pharmacological Na^+Ca^{2+} exchanger inhibition, as the defective excitation-contraction coupling in heart failure is characterized by a shift in the Ca^{2+} handling from the sarcoplasmic reticulum Ca^{2+} uptake to the sarcolemmal Ca^{2+} extrusion via the Na^+Ca^{2+} exchanger, it can be anticipated that the pharmacological inhibition of the Na^+Ca^{2+} exchanger, which decreases the Ca^{2+} influx and Ca^{2+} efflux with a concomitant increase in the sarcoplasmic reticulum Ca^{2+} transport, may be a useful mechanism in the therapy of heart failure.

Summary: conclusions and potential significance

As involvement of the Na^+Ca^{2+} exchanger in pathophysiology of cardiac disorders have been suggested by recent studies, an emerging interest can be seen in using potent and selective Na^+Ca^{2+} exchanger inhibitors as research tools, in order to clarify the detailed role of the Na^+Ca^{2+} exchanger in physiological as well as pathological conditions and to explore the possible therapeutic value of the Na^+Ca^{2+} exchanger inhibitors. Our first aim in this work was to compare the inhibitory properties of SEA0400 and KB-R7943, the two most commonly used Na^+Ca^{2+} exchanger inhibitors. We conclude that SEA0400, at sub-micromolar concentrations, can be effectively used in the Na^+Ca^{2+} exchanger research, as it blocks the exchanger current selectively and with higher potency than KB-R7943.

We have also investigated the possible interaction between the intracellular Ca^{2+} level and the inhibitory effect of SEA0400, and our results demonstrate that the hypothesized reverse Ca^{2+} -dependent inhibition of the Na^+Ca^{2+} exchanger can indeed occur in adult cardiac myocytes, with ionic concentrations being in the physiological range. Reverse Ca^{2+} -dependent inhibition by SEA0400 of the Na^+Ca^{2+} exchanger can have practical importance from therapeutic point of view, as it may provide an inherent limiting mechanism against the excessive blocking of the exchanger, thereby avoiding dangerous elevation of the intracellular Ca^{2+} level and the consequent increase in the proarrhythmic risk, which is a serious problem in the conventional therapy of heart failure.

By application of SEA0400 we are first to compare the consequences of selective pharmacological inhibition of the cardiac Na^+Ca^{2+} exchanger in intact rat and dog cardiac myocytes, showing a significant positive inotropic effect in rat myocytes. These species differ from each other substantially regarding the characteristics of their action potential waveform, intracellular Na^+ concentration and Ca^{2+} homeostasis, and it is likely that the differing effect of SEA0400 can be attributed mainly to these differences, as the mechanism of action of SEA0400 may have strong dependence on the intracellular ionic milieu. This phenomenon may possess advantages in the therapy of heart failure, as augmentation of the exchanger inhibitory effect can be expected in diseased heart with elevated intracellular Na^+ concentration. A further possible advantage of the Na^+Ca^{2+} exchanger inhibition over the conventional positive inotropic therapy is the concomitant reduction of the depolarizing inward current carried by the forward mode exchanger, which reduces the proarrhythmic risk associated with the elevated Ca^{2+} level during the therapy of heart failure.

In rat cardiac myocytes, we have also demonstrated the autoregulative nature of the Ca^{2+} homeostasis, indicating that SEA0400, as a selective pharmacological tool, may be useful in

studying the cellular Ca^{2+} homeostasis. The observed change in the Ca^{2+} fluxes further supports the possibility that inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger can exert a beneficial effect on the defective Ca^{2+} homeostasis in heart failure, as it can shift the Ca^{2+} flux balance toward an increased Ca^{2+} sequestration into the sarcoplasmic reticulum, resulting in elevated Ca^{2+} transients and cell contraction.

Although the potential application of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitors in human therapy requires further detailed studies, our work suggests that such a mechanism of action, with its advantageous dependence on the intracellular ionic environment of the diseased heart, may possess therapeutic importance in the future.

Acknowledgements

I am very grateful to **Professor Julius Gy. Papp, MD, DSc. Academician**, for his continuous support and for providing me the opportunity for research at the Department of Pharmacology and Pharmacotherapy and at the Division of Cardiovascular Pharmacology, Hungarian Academy of Sciences.

I am especially thankful to my supervisors **Professor András Varró, MD, Dsc.**, for his continuous support and personal guidance at the Department of Pharmacology and Pharmacotherapy and at the Division of Cardiovascular Pharmacology, and **András Tóth, PhD**, for introducing me to the cellular fluorescent methods. Their personal guidance and the helpful discussions were exceptionally useful during my work and allowed me to learn the critical thinking in the scientific field.

I wish to thank my colleagues, **László Virág, PhD**, and **Norbert Jost, PhD**, for their continuous support and help to study the experimental techniques in the field of the electrophysiology.

I am also very thankful to **Zsuzsanna Mohár, Gyula Horváth, Magdolna Csomor, Éva Szabadi, Gabriella Wanner** and **Gábor Girst** for their helpful technical assistance.

Finally, I wish to thank the indispensable help and love of the whole of my family, to whom I dedicate this PhD work.