Pharmacological modulation of the cardiac Na^+/Ca^{2+} exchanger: role in the study of Ca^{2+} handling and possible therapeutic applications

PhD Thesis

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1. 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 Szentandrássy, Péter P Nánási, Julius Gy Papp, András Varró, András Tóth: Effect of partial blockade of the Na^+/Ca^{2+} exchanger on Ca^{2+} handling in isolated rat ventricular myocytes.
European Journal of Pharmacology, In press, 2007.
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II. Péter Birinyi, Károly Acsai, Tamás Bányász, András Tóth, Balázs Horváth, László Virág, Norbert Szentandrássy, János Magyar, András Varró, Ferenc Fülöp, Péter P Nánási: Effects of SEA0400 and KB-R7943 on Na^+/Ca^{2+} exchange current and L-type Ca^{2+} current in canine ventricular cardiomyocytes.
Naunyn-Schmiedebergs 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

András Tóth, Károly Acsai, Attila Kun, László Virág, Julius Gy Papp, András Varró: The effect of partial Na^+-Ca^{2+}-exchanger blockage on Ca^{2+}-handling in isolated rat cardiomyocytes.
Journal of Molecular and Cellular Cardiology, 39, 185-185, 2005.

Péter Biliczki, Zsolt Nagy, László Virag, 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.

2. Introduction

2.1. Role of Na^+/Ca^{2+} exchanger in the cardiac myocytes

The Na^+/Ca^{2+} 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 (Reuter and Seitz, 1968) by measuring the dependence of Ca^{2+} efflux from cardiac muscle on ionic composition. Later it was determined that it exchanges 3 Na^+ for 1 Ca^{2+} (Reeves and Hale, 1984), which implies that its operation is electrogenic, i.e. results in electrical current flowing across the membrane (Kimura et al., 1986). The direction of this current corresponds to the mode of operation of the Na^+/Ca^{2+} exchanger. In forward mode operation (Ca^{2+} efflux), an inward depolarizing current flows into the cell, while the reverse mode (Ca^{2+} influx) results in a concomitant outward current. Resulting partly from its electrogenic property, activity of the Na^+/Ca^{2+} exchanger has a strong dependence on the instantaneous value of the membrane potential. Due to this and to the strong dependence of the Na^+/Ca^{2+} exchanger activity on the electrochemical gradients of the transported ions (DiFrancesco and Noble, 1985), operation of the Na^+/Ca^{2+} exchanger follows a complicated time course during the cardiac cycle (Luo and Rudy, 1991). In addition, besides influencing the thermodynamic driving forces, it has been shown that intracellular Na^+ and Ca^{2+} also regulate the Na^+/Ca^{2+} exchanger in an allosteric manner (Miura and Kimura, 1989; Hilgemann et al., 1992; Weber et al., 2001), making the understanding of its in situ regulation and activity exceptionally difficult. From structural point of view, the Na^+/Ca^{2+} 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^+/Ca^{2+} exchanger by Na^+ and Ca^{2+} (Nicoll et al., 1999; Matsuoka et al., 1997).

In cardiac myocytes, function of the Na^+/Ca^{2+} exchanger belongs to the most important mechanisms of the Ca^{2+} homeostasis, and displays a major contribution to the regulation of the Ca^{2+} level during the cardiac excitation-contraction coupling. The major source of Ca^{2+} triggering the Ca^{2+} cycle is the L-type Ca^{2+} current which flows into the cell at the beginning of the action potential. In this phase of the action potential, the Na^+/Ca^{2+} exchanger can

contribute to the Ca^{2+} influx (reverse mode operation) into the cell since the membrane potential is positive and the intracellular Ca^{2+} level is low. However, when the intracellular Ca^{2+} level increases at the beginning of the Ca^{2+} transient due to the Ca^{2+}-induced release of Ca^{2+} from the sarcoplasmic reticulum, the Na^+/Ca^{2+} exchanger turns into its forward mode operation, thereby contributing to the extrusion of Ca^{2+} from the cell. Relaxation of the Ca^{2+} transient is a result of the activities of tree competing mechanisms (Bers, 2001). 70-90 % of the cytoplasmic Ca^{2+} is resequestrated into the sarcoplasmic reticulum by the sarcoplasmic reticulum Ca^{2+}-ATPase, 7-30 % is extruded from the cell by the Na^+/Ca^{2+} exchanger, and only a small amount of Ca^{2+} is extruded by the slow Ca^{2+}-transport systems, such as the sarcolemmal Ca^{2+}-ATPase and the mitochondrial Ca^{2+}-transport. The Ca^{2+} balance of the cell requires that in steady state equilibrium, the beat-to-beat Ca^{2+} influx and efflux must be equal in the wide range of physiological conditions to avoid Ca^{2+} loss or Ca^{2+} overload in the cell, which means that the main transmembrane Ca^{2+} fluxes in both directions need to be finely regulated. The general outline of the function of Na^+/Ca^{2+} 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^+/Ca^{2+} exchanger in the initiation of Ca^{2+} induced Ca^{2+} release and thus the systolic Ca^{2+} transient and cell contraction (Weisser-Thomas et al., 2003). Similarly, although the crucial role of the forward mode Na^+/Ca^{2+} exchanger in extrusion of Ca^{2+} from the cell is generally accepted, its relative contribution to the total extruded Ca^{2+} can show significant variations depending on species and physiological/ pathological situations (Bassani et al., 1994; Bers, 2000). Furthermore, there are emerging hypotheses regarding some unexplored roles of the Na^+/Ca^{2+} exchanger, such as its suggested function in buffering the cytoplasmic Ca^{2+} movements (Hilgemann, 2004). Due to its crucial importance in the Ca^{2+} handling and the unresolved questions regarding its regulation, the role of Na^+/Ca^{2+} exchanger in the cardiac cycle under physiological and pathological conditions has been extensively studied in the recent years (Reuter et al., 2005; Dipolo and Beauge, 2006).

2.2. Pharmacology of the cardiac Na^+/Ca^{2+} 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^+/Ca^{2+} exchanger because of the lack of potent and highly specific inhibitors. Therefore, although there are several pharmacological agents inhibiting the Na^+/Ca^{2+} exchanger, the interpretation of results obtained using these compounds is complicated by the concomittant effects on other transport systems or ionic channels. For example, amiloride analogs have been used to study Ca^{2+} homeostasis in cardiac preparations (Brown et al., 1991), but these agents have also been shown to block the voltage gated Ca^{2+} channels, making the interpretation of their effects regarding the role of Na^+/Ca^{2+} exchanger in Ca^{2+} handling rather difficult. Other pharmacological agents such as bepridil and amiodarone (used as antiarrhythmics) or tetracaine (used as local anesthetic) are also able to inhibit the Na^+/Ca^{2+} exchanger, but again, these molecules are nonselective and the concentrations at which they inhibit the Na^+/Ca^{2+} exchanger are often even higher than the concentrations at which they exert their primary actions (Watanabe et al., 2006). The exchanger inhibitory peptid (XIP), which resembles a calmodulin binding domain, has also been used to block the Na^+/Ca^{2+} exchanger current, but its application is limited in physiological experiments because the peptide must be applied intracellularly and it also inhibits other Ca^{2+} transport systems (Enyedi and Penniston, 1993). Several trivalent and divalent cations are also capable to inhibit the Na^+/Ca^{2+} exchanger, among which Ni^{2+} has been used extensively to identify the Na^+/Ca^{2+} exchanger current in electrophysiological experiments. The main advantage of using Ni^{2+} is that it blocks Na^+/Ca^{2+} exchanger totally in a reversible manner (Hinde et al., 1999). In general, these nonselective molecules and ions can only be used in subcellular systems or in experiments in which the Na^+/Ca^{2+} exchanger is isolated by means of applying other blockers and ionic substitutions.

Therefore, the recently developed potent and selective Na^+/Ca^{2+} exchanger inhibitors present a great advance in the field of the Na^+/Ca^{2+} exchanger research, as by using these molecules the role of the Na^+/Ca^{2+} exchanger can be investigated in intact cells or tissues under physiological circumstances (Watanabe et al., 2006; Bouwman et al., 2006; Iwamoto, 2004a). The two most selective and widely used Na^+/Ca^{2+} exchanger inhibitors (see chemical structures in Fig. 1) in the literature are the aniline derivative SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxy-aniline) and the the isothiourea

derivative KB-R7943 (2-[2-[4-94-nitrobenzyloxy)phenyl]ethyl]isothiourea) (Tanaka et al., 2002; Matsuda et al., 2004 and Watanabe et al., 2006). When examining the role of the Na^+/Ca^{2+} exchanger in physiological as well as pathological circumstances by using pharmacological tools, it is important to know the selectivity, potency and possible direction dependence of the inhibitors. Therefore, the first aim of the present work was to compare the pharmacological profile of the two recently introduced Na^+/Ca^{2+} exchanger inhibitor, KB-R7943 and SEA0400.

An early study investigating the pharmacological effect of KB-R7943 concluded that this compound affects the Na^+/Ca^{2+} exchanger in a Ca^{2+} dependent manner under certain experimental circumstances (Watano and Kimura, 1998), suggesting that an interaction might exist between the drug and the intrinsic regulatory mechanisms of the Na^+/Ca^{2+} exchanger. Similar interaction has been suggested to be involved in the mechanism of action of SEA0400, as its blocking effect was found to be dependent on the intracellular Na^+ concentration, and related inversely to the intracellular Ca^{2+} concentration (Lee et al., 2004; Bouchard et al., 2004). However, these experiments were carried out in an expressed system using non-phsyological ionic concentrations to measure the Na^+/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.

2.3. Therapeutic potential of the Na^+/Ca^{2+} exchanger inhibitors

In the recent years, several studies indicated that inhibition of the Na^+/Ca^{2+} exchanger can be beneficial in experimental models of cardiac disorders. Most of these studies examined the involvement of the Na^+/Ca^{2+} exchanger in the ischemic damage of the myocardium. Based on the thermodynamics of the operation of the Na^+/Ca^{2+} exchanger (DiFrancesco and Noble, 1985), intracellular accumulation of Na^+ in ischemic/reperfused myocardium favors the reverse mode operation of the Na^+/Ca^{2+} exchanger, which may contribute to the intracellular accumulation of Ca^{2+} leading to cell damage and death (Imahashi et al., 1999; Murphy et al., 1999). Therefore, it can be speculated that pharmacological inhibition of the Na^+/Ca^{2+} exchanger may display cardioprotective effects

under such circumstances. In accordance with this hypothesis, it has been reported that SEA0400 (Takahashi et al., 2003) and KB-R7943 (Motegi et al., 2007; Seki et al., 2002) reduced infarct size in rat and rabbit (Magee et al., 2003) hearts after ischemia/reperfusion. The beneficial effects exerted by the Na^+/Ca^{2+} exchanger inhibitors in ischemia/reperfusion were also demonstrated in large animal models such as dog (Takahashi et al., 2004) and pig (Inserte et al., 2002). The above and other studies (Hagihara el al., 2005; Namekata et al., 2005 and 2006; Feng et al., 2006) also examined the proposed mechanisms of the observed protective effects of the the Na^+/Ca^{2+} exchanger inhibitors in such circumstances. Indeed, it has been verified that the most likely mechanism of action was the prevention of the excessive Ca^{2+} overload by inhibiting the reverse mode operation of Na^+/Ca^{2+} exchanger. The possible therapeutic potential of Na^+/Ca^{2+} exchanger inhibitors in ischemia-reperfusion injury was recently reviewed by Lee et al. (Lee et al., 2005).

Forward mode operation of Na^+/Ca^{2+} exchanger may also be related to pathological events, like early and delayed afterdepolarizations, in the heart when the intracellular Ca^{2+} 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 (Nagasawa et al., 2005). We also found that SEA0400 effectively decreased the occurrence of early and delayed afterdepolarizations in canine cardiac tissues (Nagy et al., 2004). The role of Na^+/Ca^{2+} exchanger in such circumstances and the possible antiarrhythmic potential of its inhibitors were reviewed by Sipido et al. (Sipido et al., 2006).

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 Na^+/Ca^{2+} exchanger inhibitors, relatively few works investigated directly the effects of the reduced Na^+/Ca^{2+} exchanger function on the Ca^{2+} homeostasis itself. KB-R7943, XIP or non-pharmacological tools were used in these studies (Satoh et al., 2000; Hobai et al., 2004; Tadros et al., 2002), 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 Na^+/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 (Eisner, 2000).

3. Major experimental goals

1. Comparison of the Na^+/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 Na^+/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 Na^+/Ca^{2+} exchanger inhibition by SEA0400 on Ca^{2+} cycling in rat cardiac myocytes by applying the perforated patch clamp technique.

4. Materials and methods

4.1. 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.

4.2. Isolation of dog cardiac myocytes

Single canine ventricular cells were obtained from hearts of adult mongrel dogs using the segment perfusion technique. The animals (10–20 kg) were anaesthetised with i.v. injection of 30 mg/kg thiopental. After opening the chest the heart was rapidly removed and a segment of left ventricular wall was perfused through the anterior descending coronary artery using a gravity flow Langendorff apparatus. The perfusion solution was the modified MEM solution (Minimum Essential Medium Eagle, Joklik modification; Sigma, M-0518), supplemented with Ca^{2+} (1.2 mM), HEPES (10 mM), taurine (2.5 g/l), pyruvic acid (175 mg/l), ribose (750 mg/l) and titrated to pH 7.2 with 1 M NaOH. After washing out the blood, the ventricular segment was perfused with nominally Ca^{2+}-free solution for 10 min. The digestion was achieved by recirculation (approx. 12 ml/min) of the perfusion medium supplemented with Ca^{2+} (75 µM) and collagenase (0.5 mg/ml, Sigma type I) for 40 min. During the isolation procedure the solutions were gassed with oxygen and the temperature was maintained at 35°C. Finally the left ventricular wall segment was minced and gently agitated. The cells freshly released from the tissue were stored at room temperature before use. At least 60% of the cells were rod shaped and showed clear striation when the external Ca^{2+} was restored.

4.3. Isolation of rat cardiac myocytes

Hearts from Sprague-Dawley rats, weighing 200-250 g, were retrogradely perfused at 37 °C for 5 min with Krebs-Henseleit solution (containing in mM: NaCl 118.5, KCl 4, $CaCl_2$ 2, $MgSO_4$ 1.0, NaH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 11.1). The pH of this solution was 7.4 when saturated with a mixture of 95 % O_2 and 5 % CO_2. Perfusion was continued with Ca^{2+}-free Krebs-Henseleit solution for further 5 min, then the perfusate was completed with collagenase (0.05 %, type I), hyaluronidase (0.05 %) and $CaCl_2$ (200 µM) and the heart was perfused for additional 10 min. Finally the left ventricular myocardium was minced and gently agitated. The cells freshly released from the tissue were stored at room temperature before use. The storage solution contained in mM: KOH 89, glutamate 70, taurin 15, KCl 30, KH_2PO_4 10, HEPES 10, $MgCl_2$ 0.5, glucose 11, EGTA 0.5, and pH was set to 7.3 with KOH. The cells were rod shaped and showed clear striation when the external calcium was restored.

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

Na^+/Ca^{2+} exchanger current ($I_{Na/Ca}$) 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 $I_{Na/Ca}$ completely (Hinde et al., 1999). However, as Ni^{2+} also blocks several other membrane currents, these experiments have to be carried out with the other currents (Na^+, K^+, Ca^{2+} and Na^+/K^+ pump currents) already blocked by specific inhibitors (Beuckelmann and Wier, 1989). Thus after establishing the whole-cell configuration in normal Tyrode solution (containing in mM: NaCl 144, NaH_2PO_4 0.33, KCl 4.0, $CaCl_2$ 1.5, $MgCl_2$ 0.53, glucose 5.5 and HEPES 5.0, pH = 7.4), the cell was superfused with a special K^+-free bath solution (containing in mM: NaCl 135, CsCl 10, $CaCl_2$ 1, $MgCl_2$ 1, $BaCl_2$ 0.2, NaH_2PO_4 0.33, TEACl 10, HEPES 10, glucose 10, pH=7.4) 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.

Micropipettes were fabricated from borosilicate glass capillaries having resistance of 2-3 MΩ when filled with the pipette solution contained (in mM) CsOH 140, aspartic acid 75, TEACl 20, Mg-ATP 5, HEPES 10, NaCl 20, EGTA 20, $CaCl_2$ 10 (pH=7.2). $I_{Na/Ca}$ was recorded using ramp pulses (having a velocity of 100 mV/s) delivered at a rate of 0.05 Hz. The membrane was initially depolarised from the holding potential of –40 mV to +60 mV, then hyperpolarised to –100 mV, and finally the membrane potential returned to the holding potential. Outward and inward $I_{Na/Ca}$ were determined during the descending limb of the ramp at +40 and –80 mV, respectively, as indicated by the arrows in Fig. 2A,B. 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 $I_{Na/Ca}$. Thus, total $I_{Na/Ca}$ was determined at both membrane potentials as a Ni^{2+}-sensitive current by subtracting the third record from the first one. The fraction of block induced by SEA0400 or KB-R7943 was expressed as percent of total $I_{Na/Ca}$.

4.5. 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. The free Ca^{2+} concentrations were calculated using WinMaxC software (Patton et al., 2004; http: //www. stanford.edu /~cpatton/winmaxc2.html), and it was verified that the free Ca^{2+} level in all cases remained in the actual buffered range.

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

Changes in intracellular free Ca^{2+} concentration were assessed by the ratiometric fluorescence technique using Fura-2-AM. Cells were incubated with 2 μM Fura-2-AM in the storage solution for 30 min followed by at least 1 hour of deestherification. After the incubation period cells were superfused in the experimental chamber with normal Tyrode solution (see above) at 35 ºC and stimulated using an electronic stimulator (DS-R1, Fonixcomp Ltd, Hungary) at 1 or 4 Hz via a pair of platinum electrodes. The chamber was attached to the stage of an inverted fluorescent microscope (IX71, Olympus, Japan). Cells were excited at 360 and 380 nm from a xenon arc lamp (Optosource, Cairn, UK). The excitatory wavelengths were selected using a galvanometric monochromator (Optoscan, Cairn, UK) operating at 100 Hz. The emitted light was band pass filtered and directed to a photomultiplier tube. The demultiplexed optical signals (360/380) were recorded using the Acquisition Engine software (Cairn, UK). Changes in intracellular free Ca^{2+} levels were approximated by the ratio of the fluorescence intensity obtained at 360 and 380 nm excitation after correction for nonspecific background fluorescence. Cell shortening was recorded using a video edge detector system (VED-105, Crescent Electronics, Sandy, Utah, USA). Concentrations of SEA0400 applied in the superfusion solution were 0.3 μM in the experiments on rat myocytes, and 0.1, 0.3 and 1 μM in case of dog myocytes, and 5 min was allowed for development of full drug effect.

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.

4.7. 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. Composition of the pipette solution in these experiments was (in mM) CsCl 140, TEACl 20, MgATP 5, NaCl 20, HEPES 10, pH = 7.2. After establishing the whole cell configuration, conditioning depolarization pulses (200 ms in duration, from -80 to 0 mV) 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.

4.8. 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. The main advantage of this method over the whole cell patch clamp configuration for measuring the L-type Ca^{2+} current is that the run down phenomenon of the L-type Ca^{2+} current can be avoided because with perforated patch clamp a good electrical access can be achieved without dialyzing the intracellular content of the cell. Further advantage of this method when studying the Ca^{2+} cycling is that the other determinants of the intracellular Ca^{2+}

transient (Ca^{2+} release channel and the sarcolasmic reticulum Ca^{2+} pump) remain intact as well.

Cells loaded with Fura-2-AM were superfused at 35 °C with normal Tyrode solution (see above) containing 5 mM of 4-aminopyridine and 0.15 µM of $BaCl_2$ to suppress K^+ currents. Micropipettes were fabricated from borosilicate glass capillaries having resistance of 2-3 MΩ when filled with pipette solution (containing in mM: CsCl 140, TEACl 20, MgATP 5, NaCl 20, HEPES 10, EGTA 10, pH = 7.2). This pipette solution was supplemented with 220 µg/ml amphotericin B in order to develop a reasonably good electrical access to cell interior. As amphotericin B penetrated the membrane patch, the series resistance decreased gradually below 15 MΩ, which was compensated for (usually at 80%). L-type Ca^{2+} currents and the intracellular Ca^{2+} transients were evoked by a composite voltage command. First a prepulse to -40 mV was applied from the holding potential of -80 mV for 40 ms, then the membrane potential was immediately clamped to 0 mV, which was followed by a voltage ramp of 50 ms duration back to the holding potential (from 0 mV to -80 mV). This voltage command was applied in trains, each containing 100 impulses, delivered at a frequency of 4 Hz. These trains were applied twice, just before each measurement (i.e. once before, and once after the 5 min period of incubation with the vehicle or 0.3 µM SEA0400). Steady-state conditions could be achieved by the end of these trains (after 25 s). Between the trains the membrane was continuously kept at the holding potential of -80 mV in order to minimize the deterioration of the perforated cell membrane. 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 commands for 100 ms from the holding potential of -40 to +60 mV in steps of 5 mV at a frequency of 0.2 Hz. Measurements were performed using Axopatch 1D amplifier controlled by pClamp 6 software (Axon Instrument). Intracellular Ca^{2+} transients and cell shortening were recorded as described above.

4.9. Drugs

All chemicals, except SEA0400, KB-R7943 and Fura-2 AM were purchased from Sigma-Aldrich (St. Louis, MO, USA). SEA0400 was synthesized (Aibe et al,. 2000) at the

Department of Pharmacological Chemistry (University of Szeged, Szeged, Hungary), Fura-2 AM was from Molecular Probes Inc. (Eugene, Oregon, USA). SEA0400 was dissolved in dimethyl sulfoxide (vehicle) and its final concentrations were 0.1, 0.3 or 1 μM when diluted in Tyrode solution.

4.10. Data handling and statistics

Data of the percent inhibition of the total $I_{Na/Ca}$ at different concentrations of SEA0400 and KB-R7943 were fitted to the Hill equation. Original traces of Ca^{2+} transients, cell shortening, and Ca^{2+} current are shown as average of 10 consecutive recordings (obtained during the last 10 members of the train in the perforated patch clamp experimets). Changes in the control (from baseline to vehicle) and SEA0400 (from baseline to SEA0400) groups were expressed as mean percent changes ± S.E.M. in case of experiments on rat. Changes after administration of SEA0400 observed in experiments on dog mocytes were normalized to the corresponding pre-drug values and were expressed as mean ± S.E.M. Means were compared statistically between groups using Student's t test for dependent or independent groups as appropriate. Data on the Ca^{2+}-dependent blocking effect of SEA0400 were analysed using ANOVA followed by linear trend test. Statistical calculations were carried out using Statistica 6.0 (StatSoft Inc.). Differences were considered significant when P was <0.05.

5. Results

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

$I_{Na/Ca}$ was recorded as a Ni^{2+}-sensitive current using the descending limb of a ramp pulse changing slowly from +60 to –100 mV during 1.6 s. Outward and inward $I_{Na/Ca}$ were determined at +40 and –80 mV, respectively (Fig. 2A,B; for further details see Materials and methods). SEA0400 and KB-R7943 suppressed both inward and outward $I_{Na/Ca}$, i.e. the Ni^{2+}-sensitive current was significantly decreased by these compounds in a concentration-dependent manner (Fig. 2C,D). Data were fitted to the Hill equation and the results are presented in Table 1. The most striking difference between the effects of SEA0400

and KB-R7943 was the difference in their affinity: the EC_{50} value (defined by the inflexion point of the Hill plot) was lower for SEA0400 than KB-R7943 by approximately 1.5 orders of magnitude when blocking either inward or outward currents. Since full inhibition of $I_{Na/Ca}$ was not achieved under our experimental conditions, drug concentrations causing 50% reduction of the currents (indicated by the dashed lines in Fig. 2C,D) were higher than the respective EC_{50} values. These numbers are presented in the last column of Table 1. 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 μM. The maximal blockade of inward $I_{Na/Ca}$ produced by the highest concentration of KB-R7943 (50 μM) was only 44.6±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.

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

Outward as well as inward $I_{Na/Ca}$ were recorded in dog cardiac myocytes as described above. Experimental groups were constructed according to the concentrations of the free Ca^{2+} used in the patch pipettes. Calculated free Ca^{2+} concentrations in the pipette solutions were 55, 140, 500 and 1000 nM. The percent values of the $I_{Na/Ca}$ inhibition by SEA0400 applied at a concentration of 1 μM are shown in Fig. 3. 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 ± 8.3 % in 55 nM Ca^{2+} group versus 39.1 ± 6.7 % in the 500 nM Ca^{2+} group, $P< 0.05$; inward current 78.2 ± 9.3 % in 55 nM Ca^{2+} group versus 42.7 ± 4.7 % in the 500 nM Ca^{2+} group, $P< 0.05$). Regarding the outward current, the percent inhibition showed a monoton 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. On the other hand, it must be kept in mind

that, although the total concentrations of Ca^{2+} and the Ca^{2+} chelators needed to achieve a buffered free Ca^{2+} level were calculated carefully, the actual momentary Ca^{2+} level near the cell membrane cannot be predicted precisely, because the cytoplasmic surface of the cell membrane also has a significant Ca^{2+} buffering capacity, which may interact with the Ca^{2+} level at the vicinity of the Na^{+}/Ca^{2+} exchanger. Therefore, the most important finding in these experiments was the tendency itself, and only careful conclusions can be made regarding the role of the absolute values of the free Ca^{2+} concentrations.

5.3. 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. After establishing steady state contractions and Ca^{2+} transients, the perfusion was switched to the solution containing 0.3 μM of SEA0400 and a period of 5 min was allowed to develop full drug effect. Original recordings of the intracellular Ca^{2+} transients and cell shortening before and after administration of 0.3 μM SEA0400 or vehicle are presented in Fig. 4. Vehicle alone failed to affect the intracellular Ca^{2+} transient and cell shortening (Fig. 4A,C). In contrast, administration of SEA0400 at a concentration of 0.3 μM resulted in an increased amplitude of both intracellular Ca^{2+} transient and cell shortening (Fig. 4B, D). In some experiments a moderate elevation in diastolic Ca^{2+} and baseline cell length was observed after SEA0400. Effects of SEA0400 on these parameters are summarized as percent changes in Fig. 5A-D. The SEA0400-induced increase in the amplitude of Ca^{2+} transient (31.6 ± 8.8 % in SEA0400, n = 6 *versus* 8.5 ± 3.8 % in vehicle, n = 7, $P < 0.05$) and in cell shortening (45.5 ± 14.9 % in SEA0400 *versus* 2.0 ± 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± 3.3 % in SEA0400 *versus* 0.9 ± 1.4 % in vehicle, P = 0.13, for diastolic Ca^{2+}; and 5.5 ± 2.9 % in SEA0400 *versus* 3.0 ± 0.8 % in vehicle, P = 0.22, for baseline cell length).

Since the relaxation kinetics of the Ca^{2+} transient may reflect kinetic properties of Ca^{2+} handling, the decay time constant of the Ca^{2+} transients was determined using monoexponential fit. No significant changes were observed in this parameter in the presence

of either SEA0400 (67.4±5.9 ms before and 72.0±2.4 ms after, n=6) or vehicle (78.1±5.7 ms before and 78.5±4.3 ms after, n=7).

5.4. Effect of SEA0400 on the intracellular Ca^{2+} transient and cell shortening in field stimulated dog cardiac myocytes, and contribution of the Na^+/Ca^{2+} exchanger to the extrusion of Ca^{2+} from the cell

Dog cardiac myocytes were stimulated at a constant frequency of 1 Hz through a pair of platinum electrodes. After establishing steady state contractions and Ca^{2+} transients, the perfusion was switched to the solution containing 0.1, 0.3 or 1 μM of SEA0400. Original recordings of the intracellular Ca^{2+} transients and cell shortening before and after administration of SEA0400 are presented in Fig. 6. Irrespective to the applied concnetrations, SEA0400 failed to affect significantly the intracellular Ca^{2+} transient and cell shortening (Fig. 6A,B). The results are summarized as changes normalized to the corresponding pre-drug values and shown in Fig. 7A,B. SEA0400 did not exert any consequent effect with the increasing concentrations, and there were no any group with statistically significant change.

To investigate why partial inhibition of the Na^+/Ca^{2+} exchanger produced no apparent effect on the Ca^{2+} handling in dog myoctes, we estimated the relative contributions of the different Ca^{2+} removal systems to the extrusion of Ca^{2+} from the cell. In patch clamped dog cardiac myocytes release of Ca^{2+} from sarcoplasmic reticulum was induced by rapid application of 10 mM caffeine after a conditioning train of depolarizations. As caffeine also prevents the accumulation of Ca^{2+} into the sarcoplasmic reticulum, the relative contribution of the remaining Ca^{2+} extrusion systems can be estimated in this experimental setting. Normalized traces of the intracellular Ca^{2+} and the parallel current recordings are shown in Fig. 8. In control conditions (Fig. 8. open symbols), the observed relaxation kinetics of the caffeine induced Ca^{2+} transient could be attributed to the combined action of the forward mode Na^+/Ca^{2+} exchanger, which generated an inward current, and other systems such as the sarcolemmal Ca^{2+} pump, and half relaxation time was 1730 ms. However, after blocking the Na^+/Ca^{2+} exchanger totally by 10 mM Ni^{2+}, which was evident by the disappearence of the inward exchanger current (Fig. 8. closed symbols), the relaxation slowed substantially,

requiring 8260 ms for half relaxation. Therefore, inactivation of the Na^+/Ca^{2+} exchanger resulted in an 4.8 -fold increase in the relaxation rate.

We investigated further the effects of Na^+/Ca^{2+} exchanger function on the intracellular Ca^{2+} transient in dog cardiac myocytes, by utilizing the strong dependence of the exchanger on the electrochemical gradient of Na^+. We recorded the intracellular Ca^{2+} transient in field stimulated dog myocytes before and after changing the concentration of extracellular Na^+ from 144 to 70 mM. As shown in Fig. 9., the decrease of the extracellular Na^+ resulted in a significant elevation of the amplitude of the systolic Ca^{2+} transient.

5.5. Effect of SEA0400 on the intracellular Ca^{2+} transient, cell shortening, and L-type Ca^{2+} current in patch clamped rat cardiac myocytes

In these experiments we aimed to reproduce the effects of SEA0400 on the intracellular Ca^{2+} transient and contractility obtained in the field stimulated rat cells, and to record L-type Ca^{2+} current simultaneously under perforated patch clamp conditions. Myocytes were paced through the patch pipette by applying trains of composite voltage commands at 4 Hz, as previously described in the methods section. Fig. 10A-C show original recordings of intracellular Ca^{2+} transients, cell shortening, and L-type Ca^{2+} current measured simultaneously in the voltage clamped cells. Recordings were made under control conditions and after application of 0.3 μM SEA0400 or vehicle. Similar to results obtained in the field stimulated cells, SEA0400 increased the amplitude of Ca^{2+} transients (32.2 ± 4.7 % in SEA0400, n = 5 *versus* -11.2 ± 6.9 % in vehicle, n = 4; $P < 0.05$) and enhanced cell shortening (25.9 ± 5.4 % in SEA0400 *versus* 0.9 ± 6.8 % in vehicle, $P < 0.05$) under voltage clamp conditions as well. In addition, both the amplitude of the L-type Ca^{2+} current (-24.9 ± 5.0 % in SEA0400, n = 5, *versus* -6.5 ± 6.7 % in vehicle, n = 4, $P < 0.05$) and the time required to half inactivation of the current (-7.9 ± 1.7 % in SEA0400, n = 5, *versus* -2.0 ± 1.5 % in vehicle, n = 4, $P < 0.05$) were significantly decreased by SEA0400. Effects of SEA0400 on the parameters above are summarized as percent changes in Fig. 11A-D indicating that the effect of SEA0400 was statistically significant in the case of each monitored parameter when compared to effects of the vehicle.

A crucial point in these experiments was the decrease in the L-type Ca^{2+} current, which was a hypothetical consequence of the elevated Ca^{2+} transients. In order to verify that SEA0400 itself did not inhibit the L-type Ca^{2+} current at the applied concentration of 0.3 μM, we recorded the L-type Ca^{2+} current in whole cell patch clamp configuration. In this experimental setting, 10 mM EGTA applied in the pipette solution effectively buffered the intracellular Ca^{2+} transient. An original recording of the L-type Ca^{2+} current and the voltage-current characteristic are presented in Fig. 12. Application of SEA0400 affected neither the amplitude nor the voltage-current characteristic of the L-type Ca^{2+} current.

6. Discussion

6.1. Inhibition of the Na^+/Ca^{2+} exchanger by SEA0400 and KB-R7943 and its relation to their pharmacological effects

We have compared the blocking effects of SEA0400 and KB-R7943 on the Na^+/Ca^{2+} exchanger current in canine ventricular myocytes, a preparation which most resembles human cardiac cells in electrophysiological properties. Regarding the blockade of the Na^+/Ca^{2+} exchanger current, three major differences were observed. First, the EC_{50} values were higher by 1.5 orders of magnitude for KB-R7943 than for SEA0400. Secondly, KB-R7943 at concentrations higher than 3 μM blocked outward $I_{Na/Ca}$ more strongly than inward $I_{Na/Ca}$, in contrast with SEA0400. Finally, the Hill coefficient obtained for SEA0400 was close to 1, whereas it was around 2 in the case of KB-R7943. These differences suggest that the Na^+/Ca^{2+} exchanger blockade induced by the two agents may involve different mechanisms. Comparing the EC_{50} values of these inhibitors for the Na^+/Ca^{2+} exchanger current and the L-type Ca^{2+} current (Birinyi et al., 2005), it can be concluded that low concentrations of SEA0400 display good selectivity for Na^+/Ca^{2+} exchanger over L-type Ca^{2+} current in canine ventricular cells, having EC_{50} values for these transporters 0.11 and 3.6 μM respectively. These results suggest that submicromolar concentrations of SEA0400 may be effectively applied to study the role of Na^+/Ca^{2+} exchanger in Ca^{2+} handling. In contrast to SEA0400, KB-R7943 had a similar potency to block the Na^+/Ca^{2+} exchanger current and the L-type Ca^{2+} current (3.3 and 3.2 μM, respectively). Due to this, and due to the higher efficacy to

block the outward than the inward exchanger current, KB-R7943 can be considered as a peculiar Ca^{2+} entry blocker having a dual mechanism to reduce Ca^{2+} influx.

Comparing our results obtained in canine ventricular cells with those of Tanaka et al. (Tanaka et al., 2002) obtained in guinea-pig myocytes, significant differences are seen. Tanaka et al. observed complete block of the Na^+/Ca^{2+} exchanger current at higher concentrations of SEA0400 and KB-R7943, and no difference was observed between the inward and outward current block induced by KB-R7943. Although the exact EC_{50} values were not reported in that study, the half-maximal block of the exchanger current was achieved at lower drug concentrations (with both SEA0400 and KB-R7943) in guinea-pig than canine ventricular cells. It is not clear at present whether differences in the effects of SEA0400 and KB-R7943 in guinea pig and dog represent true interspecies differences between the two preparations or are due to differences in the experimental conditions. Mammalian ventricular preparations are known to express only one single isoform of the Na^+/Ca^{2+} exchanger (NCX1); thus, the differences cannot be explained by contributions of different Na^+/Ca^{2+} exchanger isoforms in the two species. It is worthy of mention, however, that NCX1 is characterised by a cluster of six exons coding for a variable region in the C-terminus of the polypeptide, and alternative splicing of these exons may generate multiple tissue-specific variants of NCX1 (Quednau et al. 1997). Differences in splicing might cause differences in drug sensitivity, as single mutations were shown to modify the effect of KB-R7943 on Na^+/Ca^{2+} exchanger (Iwamoto et al. 2001; Shigekawa et al. 2002).

In some recent studies both SEA0400 and KB-R7943 were reported to prevent or reduce ischaemia/reperfusion-induced cellular injuries and arrhythmias in various mammalian cardiac preparations (Yamamura et al. 2001; Takahashi et al. 2003); however, the opposite was found by Miyamoto et al. (2002) in dog. Furthermore, KB-R7943 was also effective against ouabain-induced arrhythmias in guinea-pig (Watano et al. 1999), while SEA0400 (1 μM) suppressed early afterdepolarisations in papillary muscles and delayed afterdepolarisations in Purkinje fibre preparations of the dog (Nagy et al. 2004). The underlying mechanisms are believed to involve the reduction of Ca^{2+} entry via the reverse mode operation of the exchanger during reperfusion (Watano et al. 1999) and inhibition of the forward mode operation, that generates an inward current thought to induce afterdepolarisations under pathological conditions (Nagy et al. 2004). In the first case, a

reverse over forward mode selectivity of the Na^+/Ca^{2+} exchanger blockade was postulated with KB-R7943 (Watano et al. 1996). This was exactly the case in the present study with higher concentrations of KB-R7943; however, other investigators (Kimura et al. 1999; Lu et al. 2002) failed to demonstrate this direction-dependent selectivity. Therefore, based on the similar EC_{50} values, it is possible that prevention of Ca^{2+} overload by 5 μM KB-R7943 in strophantidin-treated rat myocytes (Satoh et al. 2000) is due not only to its blocking effect on the reverse Na^+/Ca^{2+} exchanger, but also to the inhibition of the L-type Ca^{2+} current. Similarly, the negative inotropic action of 3 μM KB-R7943 in isolated canine heart (Kurogouchi et al. 2000) might be best explained by the concomitant suppression of L-type Ca^{2+} current. Combined suppression of the Na^+/Ca^{2+} exchanger and the L-type Ca^{2+} current is not a unique feature of KB-R7943, but has been observed with several antiarrhythmic agents, such as amiodarone (Watanabe and Kimura, 2000), bepridil (Watanabe and Kimura, 2001) or aprindine (Watanabe et al., 2002), and its contribution to the antiarrhythmic potency of these compounds has to be considered. Whatever the mechanism of the prevention of the intracellular Ca^{2+} overload, the concomitant suppression of the Na^+/Ca^{2+} exchanger and the L-type Ca^{2+} current may be therapeutically beneficial, since due to the parallel reduction of transsarcolemmal Ca^{2+} influx and efflux, Ca^{2+} balance can be achieved at a lower cost of metabolic energy. In contrast, a more selective compound devoid of any effect on the L-type Ca^{2+} current at least at lower concentrations, such as SEA0400, must be used for studying the role of the Na^+/Ca^{2+} exchanger itself in physiological as well as pathological conditions.

6.2. Inverse Ca^{2+}-dependent inhibition of the Na^+/Ca^{2+} exchanger by SEA0400

It has been suggsted that SEA0400 exerts its inhibitory action by modifying the intrinsic regulatory machinery of the Na^+/Ca^{2+} exchanger. Using site directed mutagenesis, Iwamoto et al. (Iwamoto et al., 2004) pointed out that several amino acid residues in the region of the cytoplasmic loop of the Na^+/Ca^{2+} exchanger are critical for the inhibitory effect of SEA0400. On the other hand, it is well known that the cytoplasmic loop of the exchanger, possessing the exchanger inhibitory peptide (XIP) region and the regulatory Ca^{2+} binding sites, is primarily involved in various regulatory properties of the Na^+/Ca^{2+} exchanger (Matsuoka et al., 1993). Thus, if the cytoplasmic loop of the exchanger plays a central role in

both mediating the inhibitory effect of SEA0400 and in intrinsic regulatory processes of the exchanger, it is reasonable to assume that the intracellular Na^+ and Ca^{2+}, that mediate the allosteric regulation of the Na^+/Ca^{2+} exchanger, can also interact with the blocking effect of the exchanger inhibitors.

This idea is supported by experimental findings showing that inhibitory action of SEA0400 may be related to the kinetics of the Na^+-dependent inactivation (I1 inactivation) of the Na^+/Ca^{2+} exchanger (Iwamoto, 2004; Bouchard et al., 2004; Beauge and DiPolo, 2005). Although involvement of the Na^+-dependent inactivation in the beat-to-beat regulation of the Na^+/Ca^{2+} exchanger under normal physiological conditions is controversial, its existence can be clearly demonstrated in giant excised patch clamp experiments (Hilgemann, 1990). That is, when the concentration of Na^+ is elevated rapidly in the cytoplasmic surface of the membrane, the outward Na^+/Ca^{2+} exchanger current increases rapidly as well, but then it shows an inactivation which is dependent on the applied concentration of Na^+. Investigating SEA0400 in this experimental configuration Bouchard et al. (Bouchard et al., 2004) concluded that SEA0400 accelerates the Na^+-dependent inactivation of the outward exchanger current. Similarly, when analysing the effects of SEA0400 on mutant Na^+/Ca^{2+} exchanger isoforms showing accelerated or eliminated I1 inactivation, Iwamoto et al. (Iwamoto et al. 2004) also concluded that the interaction of SEA0400 with the exchanger seems to stabilize the I1 inactivation state or accelerates the rate of entry into the I1 inactive state.

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 (Lee et al., 2004), it can be expected that the intracellular Ca^{2+} can also interact indirectly with the effect of SEA0400. Indeed, investigating the Na^+/Ca^{2+} exchanger expressed in frog oocytes, Bouchard et al. (Bouchard et al., 2004) found an inverse Ca^{2+}-dependent inhibitory effect produced by SEA0400, as the percent inhibition of the total outward exchanger current was significantly decreased when the cytoplasmic free Ca^{2+} was elevated from 1 to 10 μM. 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 than that of used by Bouchard et al. (note that they activated the reverse mode operation by applying 100 mM Na^+ on the cytoplasmic surface and 8 mM Ca^{2+} on the extracellular side of the Na^+/Ca^{2+} exchanger). 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 Na^+/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 Na^+/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 Na^+/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 Na^+/Ca^{2+} exchanger is about 3-4 fold higher (Frank et al., 1992; Despa et al., 2003) 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 Na^+/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 Na^+/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 Na^+/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 Na^+/Ca^{2+} exchanger in intact cardiac myocytes, by which the dangerous Ca^{2+} overload can be avoided.

6.3. Positive inotropic effect of SEA0400 in field stimulated rat cardiac myocytes

When investigating the potential therapeutic uses of the Na^+/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 Na^+/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 Na^+/Ca^{2+} exchanger blockade depends on the relative degree of inhibition of the reverse and forward mode operation of Na^+/Ca^{2+} exchanger, as well as on the relative contribution of the two modes of Na^+/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 Na^+/Ca^{2+} exchanger (see above) and thereby decreases the Ca^{2+} influx via Na^+/Ca^{2+} exchanger, failed to affect the amplitude of either Ca^{2+} transients or contractions in rat cardiac myocytes (Satoh et al., 2000) suggesting that in this species the contribution of reverse mode Na^+/Ca^{2+} exchanger to the total Ca^{2+} influx is negligible. However, both parameters were significantly decreased by KB-R7943 in guinea pig myocytes (Satoh et al., 2003), indicating that in this species the Ca^{2+} influx via the reverse mode Na^+/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 Na^+/Ca^{2+} exchanger (see above) in patch clamped myocytes (Tanaka et al., 2002; Birinyi et al., 2005). Based on these data, in rat cardiac myocytes accentuated consequences of the forward mode blockade of the Na^+/Ca^{2+} exchanger may be anticipated with SEA0400 when comparing to results obtained with KB-R7943. 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 Na^+/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 Na^+/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 Na^+/Ca^{2+} exchanger is found predominantly in the t-tubules of the sarcolemmal membrane (Frank et al., 1992; Despa et al., 2003), 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 Na^+/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 transverse tubules, 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 Na^+/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 Na^+/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 Na^+/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 by Hobai et al. (Hobai et al., 2004) using the exchange inhibitory peptide (XIP) applied intracellularly to study the effect of the reduced Na^+/Ca^{2+} exchanger activity on Ca^{2+} handling in canine ventricular myocytes. They also observed increased amplitude of the Ca^{2+} transient without significant increase in diastolic Ca^{2+} concentration and argued that 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 (Schouten, 1990). 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.

6.4. 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 Na^+/Ca^{2+} exchanger in dog myocytes in whole cell patch clamp experiments, the possibility that SEA0400 did not inhibit the Na^+/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 Na^+/Ca^{2+} exchanger (see above), 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 (Bers, 2001). 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 Na^+/Ca^{2+} exchanger was totally inactivated by application of Na^+ and Ca^{2+} free solution, comparing to the value obtained when both the Na^+/Ca^{2+} exchanger and the slow Ca^{2+} transport systems were active (Hobai and O'Rourke, 2000). 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 Na^+/Ca^{2+} exchanger, i.e. before and after total inhibition of the exchanger by 10 mM Ni^{2+}. Our results showed that relaxation of the Ca^{2+} transient proceeded more slowly after total inhibition of the Ca^{2+} removal via the Na^+/Ca^{2+} exchanger, resulting in a 4.8-fold increase in the half relaxation time. This value is in a good agreement with those obtained by Hobai et al. (see above). However, when investigating the Ca^{2+} extruding capacity of rat and rabbit myocytes in similar experimental setting, Bassani et al. (Bassani et al., 1994) found a 8- and 10-fold increase in the relaxation time after inactivation of the Na^+/Ca^{2+} exchanger, respectively. As these values relate inversely to the Ca^{2+} extruding capacity that remains after inactivation of the Na^+/Ca^{2+} exchanger, these results indicate that in dog ventricular myocytes the Ca^{2+} removing systems other than the Na^+/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 Na^+/Ca^{2+} exchanger.

To test further the hypothesis that the similar degree of inhibition exerted by SEA0400 on both operating direction of the Na^+/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 Na^+/Ca^{2+} exchanger. That is, based on thermodynamic considerations 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 Na^+/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 Na^+/Ca^{2+} exchanger produced by SEA0400 requires the presence of intracellular Na^+, which allows the Na^+-dependent inactivation of the Na^+/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. Congruently 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 (Levi et al., 1994; Despa et al., 2002a). This possibility may have practical importance, as it is known that in heart failure the intracellular Na^+ concentration is elevated (Despa et al., 2002b). Therefore, appearance of the positive inotropic action of the Na^+/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 results from the unique mechanism of action of SEA0400, as inhibition of the Na^+/Ca^{2+} exchanger by other means explicitly results in positive inotropic effect in dog myocytes as well (Hobai et al., 2004). Therefore, we can conclude that pharmacological inhibition of the Na^+/Ca^{2+} exchanger can present a new mechanism in the positive inotropic therapy of heart failure.

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

Although SEA0400, applicated at a concentration of 0.3 µM was shown to block at least 50 % of Na^+/Ca^{2+} exchanger activity (Birinyi et al., 2005), 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 (Eisner et al., 2000), 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 µM 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 (Tanaka et al., 2002; Birinyi et al., 2005). 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 (Findlay, 2004; Zahradnikova et al., 2004). This process is mediated through calmodulin (Anderson, 2001), and provides a beat to beat basis

for an automatic negative feedback mechanism playing a key role in the autoregulation of Ca^{2+} cycling (Eisner et al., 2000). 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 Tadros et al. (Tadros et al., 2002) investigating the effects of reduced Na^+/Ca^{2+} exchanger function on the contractility and Ca^{2+} transient dynamics in rat ventricular cells came to different conclusions. Using adenovirus-mediated antisense oligonucleotid method to decrease the Na^+/Ca^{2+} exchanger expression they found that the consequences of reduced Na^+/Ca^{2+} exchanger expression on Ca^{2+} handling are largely dependent on extracellular Ca^{2+} concentration. More specifically, no significant change in the amplitude of Ca^{2+} transient at normal values (1.8 mM) of extracellular Ca^{2+} was observed in that study. 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, Tadros et al. found no change in L-type Ca^{2+} 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 (Hobai and O' Rourke, 2000 and 2004b), 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 concomittant increase in the sarcoplasmic reticulum Ca^{2+} transport, may be a useful mechanism in the therapy of heart failure.

7. 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 therapeutical 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 Na^+/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 Na^+/Ca^{2+} exchanger inhibitors in human therapy requires further detailed studies, our work suggest 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.

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