The Na\(^{+}/\)Ca\(^{2+}\) exchange blocker SEA0400 fails to enhance cytosolic Ca\(^{2+}\) transient and contractility in canine ventricular cardiomyocytes

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Received 20 June 2007; revised 16 January 2008; accepted 25 January 2008; online publish-ahead-of-print 5 February 2008

1. Introduction

The Na\(^{+}/\)Ca\(^{2+}\) exchanger (NCX) is known to be a crucial mechanism of Ca\(^{2+}\) extrusion from cardiac cells isolated from various mammalian species.\(^{1,2}\) Owing to its key role in Ca\(^{2+}\) homeostasis, agents specifically blocking NCX would be powerful tools for studies on cardiac Ca\(^{2+}\) handling. Furthermore, inhibitors of NCX were reported to prevent early and delayed after-depolarizations\(^{3,4}\) and reduce the ischaemia/reperfusion-induced cellular injuries and arrhythmias.\(^{5,6}\) The principal objection against therapeutic application of NCX blockers is based on the assumption that they elevate cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\))—as can be logically expected if NCX is the dominant mechanism of Ca\(^{2+}\) extrusion.\(^{7}\) Indeed, the selective NCX blocker SEA0400 was shown to increase [Ca\(^{2+}\)]\(_{i}\), and contractility in murine and rat myocardium.\(^{8,9}\) However, substantial differences are known to exist in Ca\(^{2+}\) handling between small rodents (mouse and rat) and larger mammalian species, including dog and human.

Recently we have shown that SEA0400 blocked effectively both inward and outward NCX currents in canine ventricular...
myocytes.\textsuperscript{10} and similar results were obtained in guinea pigs.\textsuperscript{11} Importantly, SEA0400 failed to block ion channels in the surface membrane, except for L-type Ca\textsuperscript{2\textsuperscript{+}} current.\textsuperscript{10,11} There is no information, however, on its possible actions of SEA0400 on the chief determinants of intracellular Ca\textsuperscript{2\textsuperscript{+}} handling and contractility, such as the RyR2 Ca\textsuperscript{2\textsuperscript{+}} release channel and the Ca\textsuperscript{2\textsuperscript{+}} pump in the sarcoplasmic reticulum (SR), or the Ca\textsuperscript{2\textsuperscript{+}} sensitivity of the contractile machinery. This information would be an absolute requirement for its future application either as a research tool or a therapeutic agent. Therefore, in the present work, the effects of SEA0400 on intracellular Ca\textsuperscript{2\textsuperscript{+}} handling were evaluated using isolated canine ventricular cells, a preparation most resembling human myocytes in electrophysiological properties.\textsuperscript{12,13} We found that partial blockade of NCX by SEA0400 fails to affect the [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}] transient and contractility in canine ventricular cells. This finding can well be explained by the limited inhibitory effect of SEA0400 on NCX (further attenuated by physiological [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}] levels) and the concomitant reduction in Ca\textsuperscript{2\textsuperscript{+}} influx due to the predominantly reverse mode blockade of NCX and suppression of L-type Ca\textsuperscript{2\textsuperscript{+}} current.

2. Methods

2.1 Recording of [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}], transients and cell shortening in field-stimulated myocytes

Single-canine myocytes were obtained from hearts of adult mongrel dogs of either sex, weighing 10–20 kg, anesthetized with intravenous injections of 10 mg/kg ketamine hydrochloride (Calypsol) + 1 mg/kg xylazine hydrochloride (CP-Xilazin) using the segment perfusion technique, as described previously.\textsuperscript{12} Following digestion with 1 mg/mL collagenase (Worthington, type II) in Joklik solution (St Louis, MO, USA) containing 50 μM Ca\textsuperscript{2\textsuperscript{+}} for 30 min, the left ventricular free wall was cut into small pieces and the cells were released from the tissue by gentle agitation. The viable cells were sedimented in a plexiglass chamber, allowing for continuous superfusion with oxygenized Tyrode solution containing NaCl 144, KCl 4.0, CaCl\textsubscript{2} 1.5, MgCl\textsubscript{2} 0.53, NaH\textsubscript{2}PO\textsubscript{4} 0.33, HEPES 5, and glucose 5.5 mM at pH 7.4. Intracellular [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}] transient and cell shortening were recorded as described earlier.\textsuperscript{8} Briefly, the cells were incubated with 2 μM Fura-2-AM (Molecular Probes, Eugene, OR, USA) for 30 min. After this incubation period, the cells were superfused with normal Tyrode solution at 35°C. Myocytes were stimulated using an electronic stimulator (DSS-RT, Fonixcomp Ltd, Hungary) at a constant frequency of 1 Hz through a pair of platinum electrodes. The chamber was attached to the stage of an inverted fluorescent microscope (IX71, Olympus, Japan). Cells were excited at 360 and 380 nm from a xenon arc lamp (Optosource, Cairn, UK). The excitation wavelengths were selected using a galvanometric monochromator (Optoscan, Cairn, UK) at 100 kHz switch rate. The emitted light was band-pass filtered and detected by a photomultiplier tube. The demultiplexed optical signals were recorded and analysed using the Acquisition Engine software (Cairn, UK). Changes in [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}] were characterized by the ratio of the fluorescence intensities obtained at 360 and 380 nm excitation (F\textsubscript{360}/F\textsubscript{380}) following corrections for non-specific background and bleaching. Cell shortening was recorded using a video edge detector system (VED-105, Crescent Electronics, Sandy, UT, USA) and expressed as per cent of the diastolic cell length. Original traces of Ca\textsuperscript{2\textsuperscript{+}} transient and cell shortening are presented as average of 5–10 consecutive recordings.

2.2 Recording of [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}], transients induced by caffeine

Canine ventricular myocytes were loaded with 8 μM Fluo-4 AM at room temperature for 30 min, were mounted in a low-volume imaging chamber (RC47FLP5, Warner Instruments, USA), and paced at 1 Hz. Optical measurements were performed using an Olympus IX 71 inverted fluorescence microscope. The dye was excited at 480 nm and fluorescence was measured at 535 nm by a photon counting module (H7828, Hamamatsu, Japan). Measurements were performed using the Isosys software (Experimetria, Hungary). [Ca\textsuperscript{2\textsuperscript{+}}\textsuperscript{i}] transients were induced by applying 5 μL caffeine (10 mM) containing Tyrode solution for 6 s at a flow rate of 50 μL/min directly onto the cell surface from a micropipette. These micropipettes, having typical tip diameter of 100 μm, were positioned by a manipulator to ~100 μm distance from the cell. The control caffeine flush was repeated after 2 min, then 1 μM SEA0400 was superfused for 5 min, and the caffeine fluxes were applied again. The 2 min period of time spent between the consecutive fluxes was sufficient for the cells to recover from the caffeine transient, i.e. the two subsequent challenges yielded close to identical responses, otherwise data were discarded. The two control and two SEA0400 curves were corrected against dark current, non-specific background, and bleaching artefact, averaged, and normalized so as to have identical amplitudes. When studying the effect of Ni\textsuperscript{2\textsuperscript{+}}, the cells were exposed to 10 mM NiCl\textsubscript{2} containing Tyrode for 15 s prior to the beginning of the caffeine pulse and throughout the caffeine exposure.

2.3 Measurement of Na\textsuperscript{+}/Ca\textsuperscript{2\textsuperscript{+}} exchange current in voltage-clamped myocytes

NCX current was recorded in voltage-clamped canine ventricular myocytes as described previously.\textsuperscript{10} Briefly, after establishing the whole-cell configuration in Tyrode solution, the cell was superfused with a special K\textsuperscript{+} free bath solution (containing NaCl 135, CsCl 10, CaCl\textsubscript{2} 1, MgCl\textsubscript{2} 1, BaCl\textsubscript{2} 0.2, NaH\textsubscript{2}PO\textsubscript{4} 0.33, TEACl 0.33, HEPES 10, 10 mM glucose, and pH 7.4) supplemented with 20 μM ouabain, 1 μM nisoldipine, and 50 μM lidocaine in order to block Na\textsuperscript{+}, K\textsuperscript{+}, Ca\textsuperscript{2\textsuperscript{+}}, and Na/K pump currents. The pipette solution contained CsOH 140, aspartic acid 75, TEACl 20, Mg-ATP 5, HEPES 10, NaCl 20, EGTA 20, and CaCl\textsubscript{2} 10 mM (pH 7.2). Under these conditions, Ca\textsuperscript{2\textsuperscript{+}} concentration of the pipette solution was estimated to be 140 nM using the WinMaxC software.\textsuperscript{14} Varying the CaCl\textsubscript{2} and EGTA (or in some experiments BAPTA) content of the pipette solution, free Ca\textsuperscript{2\textsuperscript{+}} values were set to 55, 140, 500, and 1000 nM, respectively. NCX current was recorded using ramp pulses (having the speed of 100 mV/s) delivered at a rate of 0.05 Hz. The membrane was initially depolarized from the holding potential of −40 to +60 mV, then hyperpolarized to −100 mV, and finally, the membrane potential returned to the holding potential. Outward and inward NCX currents were determined during the descending limb of the ramp at 40 and −80 mV, respectively, as indicated by the arrows in Figure 3A and B. After taking the control record in K\textsuperscript{+}-free solution, the cell was superfused with 1 μM SEA0400, and finally 10 mM NiCl\textsubscript{2} was added in order to fully block the current. Thus, total NCX current was determined at both membrane potentials as a Ni\textsuperscript{2\textsuperscript{+}}-sensitive current by subtracting the third record from the first one. The fraction of block induced by SEA0400 was expressed as per cent of total NCX current. Outputs from the clamp amplifier (Axopatch-2B, Axon Instruments Inc., Foster City, USA) were digitized at 20 kHz using a Digidata 1200 A/D card (Axon Instruments) and stored for later analysis, which was performed under the control of pClamp 6.0 software (Axon Instruments).

2.4 Calcium flux measurements in sarcoplasmic reticulum membrane vesicles

Heavy SR (HSR) vesicles and the ryoside receptor Ca\textsuperscript{2\textsuperscript{+}} release channel (RyR2) were isolated from canine left ventricular myocardium as described earlier.\textsuperscript{15} The vesicles were actively loaded with Ca\textsuperscript{2\textsuperscript{+}}, and the Ca\textsuperscript{2\textsuperscript{+}} efflux was determined by measuring the extravesicular Ca\textsuperscript{2\textsuperscript{+}} concentration using a Fluoromax...
spectrofluorometer (Jobin-Yvon, NJ, USA) modified for absorption measurements by monitoring the transmittance at 710 and 790 nm and calculating the corrected absorbance change (\(A_{710} - A_{790}\)) as described earlier.\(^\text{16}\) In brief, vesicles were suspended in a medium containing 92.5 mM KCl, 1 mM MgCl\(_2\), 180 \(\mu\)M antipyrilazo III (ICN Biomedicals, Aurora, OH, USA), 54 \(\mu\)M CaCl\(_2\), and 18.5 mM K-MOPS, having a final protein concentration up to 260 \(\mu\)g/mL. Vesicles were actively loaded with Ca\(^{2+}\) in the cuvette by the addition of the appropriate amount of CaCl\(_2\), and the uptake was initiated by the addition of 1 mM ATP.

The effect of SEA0400 on SR Ca\(^{2+}\) release was assessed in two ways. After completion of the ATP-ADP conversion, a second Ca\(^{2+}\) injection was applied in order to activate the release channel by adjusting the extravesicular Ca\(^{2+}\) to 20 \(\mu\)M (‘no inhibition’ protocol). SEA0400 was applied following stabilization of the rate of Ca\(^{2+}\) efflux. The rate of Ca\(^{2+}\) release was measured and compared before and after the addition of SEA0400. In the case of the ‘no activation’ protocol, the Ca\(^{2+}\) injection contained much less Ca\(^{2+}\) setting extravesicular Ca\(^{2+}\) concentration to 2 \(\mu\)M only, so the rate of baseline Ca\(^{2+}\) efflux was very low. Then SEA0400 was added to see whether there is an increase in Ca\(^{2+}\) release. Rate of Ca\(^{2+}\) efflux was calculated from the rate of change of the light intensity just prior to and after the application of SEA0400. At the end of the experiment, Ca\(^{2+}\) ionophore (2 \(\mu\)M A23187) was used to check Ca\(^{2+}\) load. Extravesicular Ca\(^{2+}\) concentration was calibrated using a very similar protocol, in the same medium without the addition of ATP, and in the presence of 2 \(\mu\)M A23187. Calibration curve was obtained by stepwise elevation of Ca\(^{2+}\) while the \(A_{710} - A_{790}\) values were recorded. Free Ca\(^{2+}\) concentrations were calculated using the absolute stability constants and the computer program developed by Fabiato and Fabiato.\(^\text{17}\)

Effect of SEA0400 on the initial rate of Ca\(^{2+}\) uptake was studied in light SR (LSR) vesicles using a similar protocol, but in this case, the vesicles were first incubated with a given concentration (0, 0.3, 1, or 3 \(\mu\)M) of SEA0400 for 15 min, then the Ca\(^{2+}\) uptake was initiated by the addition of ATP and subsequently Ca\(^{2+}\). The rate of Ca\(^{2+}\) uptake was determined by linear regression from the slope of light intensity changes using the time window between 30 and 150 s following the addition of Ca\(^{2+}\).

2.5 Planar lipid bilayer measurements

A CHAPS-solubilized single RyR2 molecule was incorporated into planar lipid bilayers.\(^\text{18}\) The bilayer was formed across a 250 \(\mu\)m aperture of a nolrene cup using a lipid mixture composed of phosphatidyl-ethanolamine, phosphatidyl-serine, and LS phosphatidyl-choline in a ratio of 5:4:1. Lipids were dissolved in \(n\)-decane up to a final concentration of 20 mg/mL. Reconstitution was initiated in symmetric buffer solution (containing 250 mM KCl, 100 \(\mu\)M EGTA, 150 \(\mu\)M CaCl\(_2\), and 20 mM PIPES, pH 7.2) by adding a small aliquot of the solubilized RyR2 to one side of the bilayer chamber defined as the cytoplasmic (cis) side. Single-channel currents were recorded at room temperature (22-25 °C) using an Axopatch 200 amplifier controlled by pClamp 6.03 software (Axon Instruments). The current was filtered at 1 kHz through an eight-pole low-pass Bessel filter and digitized at 3 kHz. Channels with conductance \(>400\) pS were accepted as RyR2. Open probability (\(P_o\)) values were calculated from minimum 2 min long segments of the current traces. After application of SEA0400 (always in the cis chamber), at least 5 min period of equilibration was allowed before the measurement.

2.6 Measurement of contractility in chemically skinned single-myocyte preparations

Small, frozen tissue blocks were first defrosted and mechanically disrupted in cell isolation solution containing MgCl\(_2\), KCl, EGTA 2, ATP 4, and imidazole 10 mM at pH 7.0. The suspension was incubated for 5 min in this solution supplemented with 0.3% Triton X-100 (Sigma), washed, and kept in cell isolation solution on ice for a maximum of 12 h. Subsequently, a demembranated single cardiomyocyte was mounted between two thin needles with silicone adhesive (Dow Corning, Midland, USA) and viewed under an inverted microscope (Axiovert 135, Zeiss, Germany) as described previously.\(^\text{19}\) One needle was attached to a force transducer element (SensoNor, Horten, Norway) and the other to an electro-magnetic motor (Aurora Scientific Inc., Aurora, Canada). Measurements were performed at 15°C, and the average sarcomere length was adjusted to 2.2 \(\mu\)m.

Composition of relaxing and activating solutions used in force measurements was calculated as described previously.\(^\text{17,20}\) The pCa values (i.e. \(-\log[Ca^{2+}]) of the relaxing and activating solutions were 9.0 ailer and 4.75, respectively. Solutions with intermediate Ca\(^{2+}\) levels were obtained by mixing activating and relaxing solutions. All solutions used for force measurements contained also MgCl\(_2\), MgATP 5, phosphocreatine 15, and N, N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid 100 mM. The ionic equivalent was adjusted to 150 with KCl at an ionic strength of 186. Isometric force was measured after the preparation had been transferred from the relaxing solution to a Ca\(^{2+}\)-containing one. When a steady level of force was reached, the length of the myocyte was abruptly (within 2 ms) reduced by 20% and then quickly re-stretched. As a result, the force first dropped from the peak isometric level to zero and then started to re-develop. The passive component of force was determined in relaxing solution following the Ca\(^{2+}\) contractures. Ca\(^{2+}\)-activated isometric force was calculated by subtracting the passive force from the peak isometric force, obtained at pCa = 4.75. After the first maximal activation at pCa = 4.75, the resting sarcomere length was re-adjusted to 2.2 \(\mu\)m, when it was necessary. This second maximal activation at pCa = 4.75 was used to calculate maximal isometric force (\(P_{\text{max}}\)). Cells were subsequently exposed to a series of solutions with intermediate pCa values. The Ca\(^{2+}\)-activated force measured at these intermediate levels of activation was normalized to \(P_{\text{max}}\) and data were fit to the Hill equation in order to estimate Ca\(^{2+}\) concentration, resulting in half-maximal force production (pCa\(_{50}\)) as shown in Figure 6A. If at the end of this series re-exposure to pCa 4.75 yielded a \(P_{\text{max}}\) <80% of the initial value, the measurement was discarded.

2.7 Statistics

Results are expressed as mean ± SEM values. Statistical significance of differences obtained between control and SEA0400 treated preparations was evaluated with Student’s t-test for paired or unpaired data, as relevant. Differences were considered significant when \(P\) was <0.05.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

3. Results

3.1 Effect of SEA0400 on [Ca\(^{2+}\)], transient and cell shortening in field-stimulated myocytes

Myocytes were stimulated at a constant frequency of 1 Hz through a pair of platinum electrodes. After establishing steady-state contractions and [Ca\(^{2+}\)], transients, the perfusion was switched to the solution containing 0.1, 0.3, or 1 \(\mu\)M SEA0400, and 5 min was allowed to develop full drug effects. Original recordings of [Ca\(^{2+}\)], transients and cell shortening before and after administration of SEA0400 are presented in Figure 1A and B. Average values obtained for the diastolic fluorescent ratio, \(F_{380}/F_{580}\) amplitude, and cell shortening are summarized in Figure 1C-E. Data indicate that no significant change in these parameters was
caused by SEA0400 in the 0.1–1 μM concentration range. Similarly, no significant change was observed in the decay time constant of the [Ca$^{2+}$]$_i$ transient determined before and after the application of 1 μM SEA0400 (269 ± 41 and 266 ± 17 ms, respectively, n = 9).

3.2 Effect of SEA0400 on [Ca$^{2+}$]$_i$ transients induced by caffeine

Since the relaxation kinetics of a [Ca$^{2+}$]$_i$ transient reflects the kinetic properties of Ca$^{2+}$ elimination from the cytosol, NCX inhibition is accompanied with the elongation of the decay of [Ca$^{2+}$]$_i$ transients induced by rapid application of caffeine. Therefore, the effect of 1 μM SEA0400 was studied on [Ca$^{2+}$]$_i$ transients evoked by superfusion with 10 mM caffeine for 6 s. In order to compare the time course of decay, the transients were normalized to identical amplitudes and superimposed (Figure 2A). The decay time constant, determined using mono-exponential fit, was significantly increased by 1 μM SEA0400 from 1.03 ± 0.05 to 1.32 ± 0.06 s (P < 0.05, n = 10), whereas the respective time-matched control values (i.e. when SEA0400 was omitted from the solution) were 0.96 ± 0.05 and 0.95 ± 0.04 s (NS, n = 8). These results directly prove that under the given conditions, SEA0400 is capable to partially inhibit NCX. The NCX-blocking potency of SEA0400 can be qualitatively evaluated by comparing with that of 10 mM NiCl$_2$, which is known to fully block the exchanger. As in the presence of NiCl$_2$ the decaying limb could not be fitted exponentially, the time of half-relaxation was determined: it was increased by NiCl$_2$ from 1.3 ± 0.1 to 10.1 ± 1.3 s (P < 0.05, n = 4). Although the effect of 1 μM SEA0400 on the rate of decay of the caffeine-induced [Ca$^{2+}$]$_i$ transient was only a fraction of that observed with 10 mM NiCl$_2$, it was clearly detectable. However, these results suggest that the NCX-blocking effect of SEA0400 may be relatively moderate when [Ca$^{2+}$]$_i$ is elevated.

3.3 Calcium-dependent effect of SEA0400 on Na$^+/Ca^{2+}$ exchange current

NCX current was recorded as a Ni$^{2+}$-sensitive current using the descending limb of a ramp pulse changing slowly from 60 to –100 mV during 1.6 s. Outward and inward NCX current was determined at 40 and –80 mV, respectively (Figure 3A and B; for further details, see Methods section). One micro mole SEA0400 suppressed both inward and outward NCX current; however, suppression of the outward current was more...
pronounced than the inward current block. The effect of SEA0400 on NCX current was studied at various levels of [Ca\(^{2+}\)], (ranging from 55 nM to 1 \(\mu\)M) set by changing the CaCl\(_2\) and EGTA content of the pipette solution. The blocking effect of 1 \(\mu\)M SEA0400 on the inward (open symbols) and outward (filled symbols) Na\(^+\)/Ca\(^{2+}\) exchange currents as a function of [Ca\(^{2+}\)]. (C). Free Ca\(^{2+}\) was set to 55, 140, 500, and 1000 nM by varying the CaCl\(_2\) and EGTA content of the pipette solution. Symbols and bars represent mean ± SEM, numbers in parentheses denote the number of cells studied.

Figure 3 Effect of SEA0400 on Na\(^+\)/Ca\(^{2+}\) exchange current as a function of [Ca\(^{2+}\)]. Ramp protocols used to elicit Na\(^+\)/Ca\(^{2+}\) exchange current (A) and superimposed current traces obtained in K\(^+\)-free bath solution (control), in the presence of 1 \(\mu\)M SEA0400, and after addition of 10 mM NiCl\(_2\) in order to fully block Na\(^+\)/Ca\(^{2+}\) exchange current (B). Outward (reverse mode) and inward (forward mode) Na\(^+\)/Ca\(^{2+}\) exchange currents were determined during the descending limb of the ramp at 40 and -80 mV, respectively, as indicated by the arrows. Blocking effect of 1 \(\mu\)M SEA0400 on the inward (open symbols) and outward (filled symbols) Na\(^+\)/Ca\(^{2+}\) exchange currents as a function of [Ca\(^{2+}\)]. (C). Free Ca\(^{2+}\) was set to 55, 140, 500, and 1000 nM by varying the CaCl\(_2\) and EGTA content of the pipette solution. Symbols and bars represent mean ± SEM, numbers in parentheses denote the number of cells studied.

3.4 Effect of SEA0400 on sarcoplasmic reticulum calcium release

In HSR vesicle preparations, the effect of SEA0400 was studied by measuring Ca\(^{2+}\) release from the vesicles in the presence of 20 \(\mu\)M extravesicular Ca\(^{2+}\). This Ca\(^{2+}\) concentration makes the RyR2 partially open as seen from the increase of extravesicular Ca\(^{2+}\) concentration in Figure 4A. Addition of SEA0400 failed to alter the rate of release as revealed by the unchanged slope of the Ca\(^{2+}\) flux, indicating that the drug did not modify Ca\(^{2+}\) flux through the partially open channel (‘no inhibition’ protocol). The rate of Ca\(^{2+}\) release was 12.4 ± 0.9 nmol/min/mg protein before the application of SEA0400, whereas it was 11.8 ± 1.1, 13.1 ± 1.2, and 13.2 ± 1.2 nmol/min/mg protein in the presence of 0.3, 1, and 3 \(\mu\)M SEA0400, respectively (n = 7 for each).

Similarly designed experiments were performed to study the effect of SEA0400 on closed RyR2 channels. In this case, lower, just subthreshold extravesicular Ca\(^{2+}\) concentrations were applied. The horizontal segment of the record indicates that the channels are practically closed. SEA0400 failed to induce any Ca\(^{2+}\) release, in contrast to the Ca\(^{2+}\) ionophore, A23187 (2 \(\mu\)M), which caused marked Ca\(^{2+}\) release, showing that the vesicles were indeed fully packed with Ca\(^{2+}\). A typical experiment performed using this ‘no activation’ protocol is depicted in Figure 4B. Under these experimental conditions, the rate of baseline Ca\(^{2+}\) release was 2.2 ± 0.31 nmol/min/mg protein before the application of SEA0400, whereas it was 2.2 ± 0.3, 2.3 ± 0.28, and 2.3 ± 0.3 nmol/min/mg protein in the presence of 0.3, 1, and 3 \(\mu\)M SEA0400, respectively (n = 6 for each).

3.5 Effect of SEA0400 on sarcoplasmic reticulum calcium uptake

Activity of the SR calcium pump was estimated by measuring the initial uptake rate of LSR vesicles by measuring time-dependent changes in extravesicular Ca\(^{2+}\) concentration under maximal pumping rate conditions. The uptake rate was determined in the absence and presence of 0.3, 1, and 3 \(\mu\)M of SEA0400 (Figure 4C and D). The initial uptake rates were identical with and without SEA0400, indicating that SEA0400 fails to modify the re-uptake of Ca\(^{2+}\) from the intracellular space to the lumen of the SR.

3.6 Effect of SEA0400 on the gating of single-RyR2 channels

Effect of SEA0400 on single-RyR2 channels was also studied using Müller-type bilayer, where the solubilized RyR2 was incorporated into the lipid membrane, and single-channel current was measured under voltage clamp conditions. According to the analogue records presented in Figure 5A, the channels were almost fully open in the presence of 50 \(\mu\)M Ca\(^{2+}\) on the cis side: their open probability (\(P_o\)) was 0.978 ± 0.047 (n = 9). Reducing cis Ca\(^{2+}\) to 472 nM channels exhibited a \(P_o\) of 0.043 ± 0.008 (n = 9). After addition of 3 \(\mu\)M SEA0400, the open probability failed to change significantly (\(P_o\) = 0.032 ± 0.005, n = 6). It is also evident from Figure 5A that the conductance of RyR2 remained unaltered in the presence of SEA0400, since amplitudes of single-channel currents were the same. Determining the mean open time of the channel revealed that two components are necessary to fit the open-time histograms with a reasonable correlation coefficient. No significant differences were observed in the faster or slower time constants estimated before and after the application of 3 \(\mu\)M SEA0400, as shown in Figure 5B. In summary, SEA0400 failed to modify
any of the characteristic parameters of RyR2 up to the concentration of 3 μM.

3.7 Effect of SEA0400 on Ca²⁺ sensitivity of contractile proteins in chemically skinned myocytes

As described in the Methods section, first the force–pCa relationship was determined by exposing the preparation to stepwise increases of external Ca²⁺ in order to determine the half-activation Ca²⁺ concentration, defined as pCa₅₀. The value estimated for pCa₅₀ in these preparations was 6.4 (Figure 6A). The maximal isometric Ca²⁺-activated force was 27.9 ± 9.8 kN/m², and the passive force was 3.6 ± 1.7 kN/m² in the four permeabilized cardiomyocytes, each obtained from a different heart. Force development was measured in the absence and presence of 1 μM SEA0400 at the single Ca²⁺ concentration of pCa = 6.4.

Therefore, any hypothetical SEA0400-dependent alteration in Ca²⁺-dependent force production was not limited by Ca²⁺ saturation of the contractile machinery. SEA0400 was dissolved in concentrated DMSO; therefore, control measurements in the presence of DMSO were also included. The Ca²⁺-activated force did not differ in the presence or absence of 3 μM SEA0400 to increase the extravesicular Ca²⁺ concentration to 10 μM. (D) Average rate of Ca²⁺ uptake determined without SEA0400 pretreatment or in the presence of 0.3, 1, and 3 μM SEA0400. Numbers in parentheses denote the number of independent determinations.

4. Discussion

In this study, we have demonstrated that—in contrast to murine and rat ventricular myocardium⁸,⁹—partial blockade of NCX by SEA0400 fails to affect the [Ca²⁺], transient and contractility in canine ventricular cells. In the absence of

Figure 4. Effect of SEA0400 on Ca²⁺ release [(A) and (B)] and Ca²⁺ uptake [(C) and (D)] in sarcoplasmic reticulum membrane vesicles. (A) Typical record of the ‘no inhibition’ protocol. After loading the vesicles with Ca²⁺ by addition of ATP in the presence of Ca²⁺, the extravesicular Ca²⁺ concentration was set to 20 μM by a second Ca²⁺ injection in order to activate RyR2 channels. The rate of release was calculated from the slope of the curve measured just before and after application of SEA0400. Light intensity, shown in the ordinate, is inversely related to extravesicular Ca²⁺ concentration. (B) The effect of SEA0400 on the resting (closed) RyR2 channels was studied using the ‘no activation’ protocol. In this case, after loading the vesicles, the extravesicular Ca²⁺ concentration was set to 2 μM. This caused only negligible activation of the channels, which was not modified by the subsequent application of SEA0400. Finally, the Ca²⁺ content of the vesicles was fully released by the Ca²⁺ ionophore A23187 (2 μM). (C) Representative pair of superimposed recordings showing the initial rate of Ca²⁺ uptake as determined from the slope of the post-Ca²⁺ segment of the light intensity signal. Ca²⁺ uptake was initiated by addition of Ca²⁺ (arrows) in the presence or absence of 3 μM SEA0400 to increase the extravesicular Ca²⁺ concentration to 10 μM. (D) Average rate of Ca²⁺ uptake determined without SEA0400 pretreatment or in the presence of 0.3, 1, and 3 μM SEA0400. Numbers in parentheses denote the number of independent determinations.
any direct effect of SEA0400 on sarcoplasmic Ca^{2+} transport and the contractile proteins (as also shown in the present study), this is only possible if the net effect of SEA0400 on transmembrane Ca^{2+} transport is close to zero (i.e. the effects on the outward and inward Ca^{2+} fluxes are approximately equal). As it was previously shown, 1 μM SEA0400 blocked more than 50% of inward NCX current when [Ca^{2+}]_{cis}, was kept relatively low.\(^{10}\) Such significant inhibition of NCX current should lead to [Ca^{2+}]_{cyt}, accumulation accompanied by increased [Ca^{2+}]_{cyt}, transients and cell shortening, which changes clearly failed to occur. On the other hand, the NCX-blocking effect of SEA0400 in canine myocytes depended on the actual level of [Ca^{2+}]_{cyt}.\(^{10}\) Suppression of NCX current was reduced to half when [Ca^{2+}]_{cyt}, was increased from 55 nM to 1 μM, resulting in a 28% of inhibition. Since [Ca^{2+}]_{cyt} is believed to reach much higher values in the subsarcolemmal fuzzy space, the blocking effect of SEA0400 on NCX may even be lower under physiological conditions. This moderate reduction of Ca^{2+} extrusion, however, still must be balanced by proportional reduction of Ca^{2+} influx in absence of inotropic response. Since 1 μM SEA0400 was shown to decrease \(I_{Ca}\) by 20% in canine ventricular cells,\(^{10}\) this balance could easily be achieved. At 0.3 μM concentration, where the SEA0400-induced \(I_{Ca}\) blockade was <5% while the inhibition of NCX was almost as strong as measured with 1 μM SEA0400,\(^{10}\) further mechanisms have to be involved in the maintenance of equilibrium. For instance, congruently with our present results, SEA0400 was shown to block the reverse mode action of NCX more effectively than its forward mode operation,\(^{22}\) which may result in the reduction of Ca^{2+} entry. The large reserve capacity of NCX, together with the activation of NCX-independent mechanisms of Ca^{2+} extrusion (via sarcosomal- and mitochondrial Ca^{2+} pump) or SR-independent Ca^{2+} sequestration (via mitochondrial Ca^{2+} uptake), may also contribute to elimination of cytosolic calcium. The signal to turn on these compensatory mechanisms is apparently missing, since neither diastolic nor systolic [Ca^{2+}]_{cyt}, was elevated by SEA0400. However, it must be borne in mind that moderate changes in subsarcolemmal [Ca^{2+}]_{cyt}, are not likely detected by the conventional [Ca^{2+}]_{cyt}-monitoring techniques. Although none of the outlined mechanisms alone seems to be sufficient to account for the inability of 0.3 μM SEA0400 to modify [Ca^{2+}]_{cyt}, transient and contractility in canine myocytes, their combined effects may likely explain our observations. The contribution of NCX-independent mechanisms to Ca^{2+} elimination is supported by the finding that relatively normal [Ca^{2+}]_{cyt}, transients could be observed in embryonic heart tubes of NCX knockout mice.\(^{23}\)

Our present results with SEA0400 apparently conflicts with the observations of Hobai et al. obtained with exchange inhibitory peptide in healthy canine heart cells.\(^{14}\) In that study, 27% inhibition of NCX resulted in approximately 50% increase in systolic [Ca^{2+}]_{cyt}. This discrepancy can likely be attributed to the pharmacological properties of SEA0400 as discussed earlier, i.e. the predominantly reverse mode NCX
inhibition combined with some suppression of \( I_{\text{Ca}} \). However, the reaction of a failing heart to NCX inhibition may also be different from normal.

As it was mentioned earlier, markedly different results were obtained in small rodents, where 1 \( \mu \text{M} \) SEA0400 increased the magnitude of both \( [\text{Ca}^{2+}] \), transients and cell shortening.\(^8,9\) This may be due to the lack of a distinct plateau phase in their action potential—in contrast to dogs and humans. Consequently, NCX spends relatively longer time in the forward mode during the cardiac cycle in rodents than in larger animals, favouring increased \( \text{Ca}^{2+} \) extrusion, which in turn may lead to increased \( [\text{Ca}^{2+}] \), transient and force development when NCX is blocked. In our canine myocytes, however, SEA0400-induced changes in \( [\text{Ca}^{2+}] \), transient and contraction were absent, suggesting that results obtained in small rodents can be interpreted for dogs or humans only with a particular care.

Since the selectivity of SEA0400 has been questioned,\(^{23}\) it is crucial to know whether SEA0400 can directly modify sarcoplasmic \( \text{Ca}^{2+} \) fluxes. Our results indicate that SEA0400 fails to alter the conductance or gating kinetics of the \( \text{RyR2} \) \( \text{Ca}^{2+} \) release channel, has no effect on the rate of sarcoplasmic \( [\text{Ca}^{2+}] \) release and re-uptake up to a concentration of 3 \( \mu \text{M} \), or \( [\text{Ca}^{2+}] \) sensitivity of the myofilaments. These results, congruent with the unchanged \( [\text{Ca}^{2+}] \), transients, indicate that SEA0400 exerts no direct action on sarcoplasmic \( \text{Ca}^{2+} \) handling in canine ventricular cells. Therefore, 0.3 \( \mu \text{M} \) SEA0400, which had negligible effects on transmembrane ion currents,\(^{10}\) can be considered a selective inhibitor of NCX, and as such, is a suitable tool to study the effects of partial NCX inhibition in cardiac myocytes.

The \( [\text{Ca}^{2+}] \), dependent action of SEA0400 on NCX, shown by us in patch-clamped canine myocytes, seems to be an important feature of the compound. Although it has already been observed in the case of outward NCX current (corresponding to the reverse mode operation of NCX) recorded from Xenopus oocytes,\(^{22,25}\) the present study is the first one to demonstrate this effect on the forward mode operation of NCX. Furthermore, our experimental conditions better approximated the physiological situation regarding the ionic composition of the extracellular and intracellular solutions applied. The \( [\text{Ca}^{2+}] \), dependent inhibitory effect of SEA0400 on NCX may be related to the SEA0400-induced acceleration of \( [\text{Na}^{+}] \) dependent inactivation of the current, which is enhanced by [\( \text{Na}^{+} \)], and reduced by \( [\text{Ca}^{2+}] \).\(^{26}\) This implicates that the effect of SEA0400 is augmented under conditions of high \( [\text{Na}^{+}] \), as it was shown to occur in Xenopus oocytes expressing NCX 1.1 isoform.\(^{22}\) These results together may explain why the NCX blocker SEA0400 is able to prevent \( \text{Ca}^{2+} \) overload under conditions of ischaemia/reperfusion (when NCX, driven by the elevated \( [\text{Na}^{+}] \), works predominantly in its reverse mode) without causing \( \text{Ca}^{2+} \) overload in healthy canine (and probably human) cardiac tissues, where NCX operates entirely in forward direction.

**Funding**


**Acknowledgements**

The authors thank Mrs Vighné Katain Horváth for the excellent technical assistance.

**Conflict of interest:** none declared.

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