Pharmacological Inhibition of Na/Ca Exchange Results in Increased Cellular Ca^{2+} Load Attributable to the Predominance of Forward Mode Block

Semir Ozdemir, Virginie Bito, Patricia Holemans, Laurent Vinet, Jean-Jacques Mercadier, Andras Varro, Karin R. Sipido

Abstract—Block of Na/Ca exchange (NCX) has potential therapeutic applications, in particular, if a mode-selective block could be achieved, but also carries serious risks for disturbing the normal Ca^{2+} balance maintained by NCX. We have examined the effects of partial inhibition of NCX by SEA-0400 (1 or 0.3 μmol/L) in left ventricular myocytes from healthy pigs or mice and from mice with heart failure ($MLP^{-/-}$). During voltage clamp ramps with $[Ca^{2+}]_i$ buffering, block of reverse mode block was slightly larger than of forward mode (by 25±5%, $P<0.05$). In the absence of $[Ca^{2+}]_i$ buffering and with sarcoplasmic reticulum (SR) fluxes blocked, rate constants for Ca^{2+} influx and Ca^{2+} efflux were reduced to the same extent (to 36±6% and 32±4%, respectively). With normal SR function the reduction of inward NCX current (I_{NCX}) was 57±10% (n=10); during large caffeine-induced Ca^{2+} transients, it was larger (82±3%). $[Ca^{2+}]_i$ transients evoked during depolarizing steps increased (from 424±27 to 994±127 nmol/L at +10mV, $P<0.05$), despite a reduction of I_{CaL} by 27%. Resting $[Ca^{2+}]_i$ increased; there was a small decrease in the rate of decline of $[Ca^{2+}]_i$. SR Ca^{2+} content increased more than 2-fold. Contraction amplitude of field-stimulated myocytes increased in healthy myocytes but not in myocytes from $MLP^{-/-}$ mice, in which SR Ca^{2+} content remained unchanged. These data provide proof-of-principle that even partial inhibition of NCX results in a net gain of Ca^{2+}. Further development of NCX blockers, in particular, for heart failure, must balance potential benefits of I_{NCX} reduction against effects on Ca^{2+} handling by refining mode dependence and/or including additional targets. (***Circ Res*. 2008;102:1398-1405.**)

Key Words: cardiac myocytes ■ heart failure ■ Na/Ca exchange ■ sarcoplasmic reticulum ■ calcium overload

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The Na/Ca exchanger has a key role in the Ca^{2+} flux balance of cardiac myocytes as it is the major Ca^{2+} efflux pathway to remove Ca^{2+} entry occurring during excitation–contraction coupling (reviewed elsewhere[1,2]). With each heartbeat, Ca^{2+} influx across the sarcolemma triggers a release of Ca^{2+} from the sarcoplasmic reticulum (SR), the major source for Ca^{2+} for contraction. Ca^{2+} influx is mainly through the voltage-dependent L-type Ca^{2+} channels (I_{CaL}) and, to a lesser extent, through the Na/Ca exchanger itself. The net transport of Ca^{2+} through the exchanger during a cycle is thus Ca^{2+} removal. Changes in flux through the exchanger occur consequent on alterations in $[Ca^{2+}]_i$ and $[Na^+]_i$ (reviewed elsewhere[3]). Such changes can affect the total amount of Ca^{2+} available in the SR for release.

The Na/Ca exchange (NCX) current (I_{NCX}) is inward for Ca^{2+} removal (forward mode) and outward for Ca^{2+} influx (reverse mode). I_{NCX} can be arrhythmogenic because the inward current during Ca^{2+} release from the SR can give rise to delayed afterdepolarizations when SR Ca^{2+} release occurs outside the normal excitation–contraction coupling cycle (reviewed elsewhere[4]). This can be attributable to Ca^{2+} overload with or without a lowering of the threshold for release because of altered properties of the ryanodine receptor.[5–7] Outward I_{NCX} can by itself contribute to Ca^{2+} loading when $[Na^+]_i$ is increased, as, for example, during Na/K pump block but also in heart failure.[8]

From the purely electrophysiological viewpoint, reducing inward I_{NCX} could theoretically be antiarrhythmic, whereas selective block of the outward I_{NCX} would reduce Ca^{2+} loading.[9–11] Several recent developments have rekindled the interest in potential benefits of blocking, or at least reducing, NCX. KBR-7943 and the more potent and selective SEA-0400 were reported to have a higher potency in blocking reverse mode than forward mode, which could be related to Na-dependent binding.[12–14] Such drugs could thus reduce Ca^{2+} overload related to reverse mode NCX. Indeed, in conditions of increased Ca^{2+} influx via reverse mode, such as block of the Na/K pump, KBR-7943 and SEA-0400 reduced

Original received May 30, 2007; resubmission received March 3, 2008; revised resubmission received April 14, 2008; accepted April 23, 2008.

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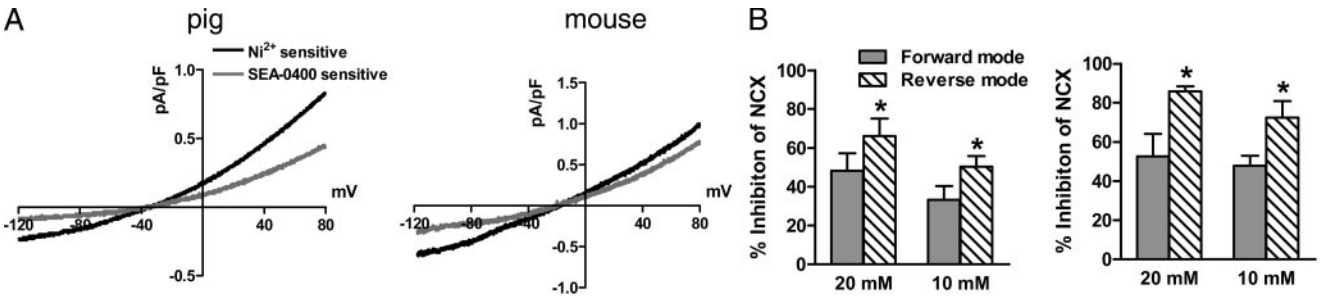


Figure 1. Block of I_{NCX} by 1 μmol/L SEA-0400. A, Averaged data (n=8 for pig, n=5 for mouse) of the total I_{NCX} during a ramp protocol (step from −40 to +80 mV, repolarizing ramp to <120 mV in 2 seconds), measured as the Ni-sensitive current, and of the current blocked by SEA-0400. $[NaCl]_{pipette}$, 10 mmol/L. B, Block by SEA-0400 is calculated from the SEA-sensitive current as a fraction of the total I_{NCX}, at 60 mV on either side of the reversal potential for $[NaCl]_{pipette}$ of 10 mmol/L (n=8 for pig, n=5 for mouse) and 20 mmol/L (n=7 for both). *P<0.05 forward vs reverse mode.

arrhythmias.[15,16] Mice with cardiac-specific knockout of the Na/Ca exchanger had a higher resistance to ischemia/reperfusion damage.[17] Despite absence of NCX, they had normal overall $[Ca^{2+}]_i$ transients, maintaining Ca^{2+} balance most likely because of a reduced Ca^{2+} influx.[18,19] However, caveats against NCX block have also been raised. Theoretical modeling and experiments with XIP predict an increase in Ca^{2+} load from a block of NCX because of its role in Ca^{2+} efflux.[20] Under conditions in which the exchanger would thus not be stimulated in its reverse mode, NCX block could have different effects. Secondly, the selective block of reverse more than forward mode by these agents is less pronounced in more physiological conditions,[21,22] although consequent changes in Ca^{2+} handling were so far not studied.

In the present study, we tested the hypothesis that in normal myocytes and physiological conditions SEA-0400 would not exhibit mode dependence and that even partial inhibition of NCX would lead to a net gain of Ca^{2+}. Our findings support this hypothesis. We also confirm the potency of this drug to reduce potentially arrhythmogenic inward NCX currents. Further development of NCX inhibitors should thus weigh the potential benefits of current block against the effects on Ca handling.

Materials and Methods

Cell Isolation

Single left ventricular (LV) myocytes were enzymatically isolated from the hearts of domestic pigs of either sex (body weight, 40 to 45 kg; N=18) as previously described.[23] We used only cells isolated from the midmyocardial layer of the left ventricle. For mice, we used LV myocytes without distinguishing layers within the left ventricle; the isolation procedure was as previously described.[24] Wild-type mice had a mixed background of 129SV/Swiss (N=13), matching the background of the $MLP^{-/-}$ mice (N=6) originally reported.[25]

Cells were stored at room temperature and used within 24 hours. The characterization of Ca^{2+} handling was done in both species, but some specific and complementary experiments were done in pig myocytes only.

Solutions and Drugs

Experiments were done in normal Tyrode solution (in mmol/L): NaCl 137, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1.8, Na-Hepes 11.8, glucose 10; pH 7.40. The pipette solution for whole-cell patch clamp contained (in mmol/L): K-aspartate 120, NaCl 10, KCl 20, K-Hepes 10, MgATP 5, K_5fluo-3 0.05; pH 7.2. To measure Ca^{2+} and NCX currents, K^+ was replaced with Cs^+ in pipette and extracellular solutions.

For characterizing block of I_{NCX} by SEA-0400 during ramp protocols, the solution consisted of (in mmol/L): NaCl 130, TEA-Cl 10, Na-Hepes 11.8, $MgCl_2$ 0.5, $CaCl_2$ 1.8, ryanodine 0.005, nifedipine 0.02, glucose 10 (pH 7.4); the pipette solution: CsCl 65, $CaCl_2$ 10.92, EGTA 20, Hepes 10, MgATP 5, $MgCl_2$ 0.5, TEA-Cl 20 (pH 7.2); and calculated free $[Ca^{2+}]$ 150 nmol/L.

A stock solution of SEA-0400 (a gift from F. Fülöp[26]) was prepared in DMSO at a concentration of 2 mmol/L. The final concentration to be used was obtained by diluting 1:2000 or 1:6000 (vol/vol) in normal Tyrode. A 1:2000 dilution of the solvent in normal Tyrode solution had no effects by itself (n_{cells}=9, data not shown). $NiCl_2$ (Sigma-Aldrich, Bornem, Belgium) was dissolved directly at 2.5 mmol/L.

Experimental Setup

The setup for fluorescence and membrane currents recording was as described before.[27] The fluorescence signals were corrected for the background recorded after the seal formation. After patch rupture, sufficient time was allowed for dye equilibration. The fluorescence signal was further calibrated to $[Ca^{2+}]_i$ values according to $F=F_{max}[Ca^{2+}]_i/([Ca^{2+}]_i+K_d)$.[28] F_{max} was obtained at the end of each experiment by strong hyperpolarization of the cell. Movement artifacts were avoided by measuring fluorescence from the entire cell.

Experimental Protocols

During voltage clamp experiments, the holding potential was −70 mV. For measuring I_{CaL} the Na^+ current was inactivated by a prepulse to −50 or −40 mV. I_{CaL} was taken as the difference between the peak inward current and the current at the end of the pulse during the subsequent depolarizing steps.

The Ca^{2+} content of the SR, I_{NCX} and NCX-dependent Ca^{2+} removal were measured during a 10-second application of 10 mmol/L caffeine via a fast superperfusion system, at a holding potential of −50 mV following a train of 10 conditioning pulses from −70 to +10 mV at 1 Hz (Figure 5). In another set of experiments, a brief application (300 ms) was used to induce SR release without inhibition of subsequent SR reuptake (Figure 6C). All the experiments were done at 37°C.

Statistics

All data are shown as means±SEM. For statistical analysis, a paired Student's t test was used for single measurements and ANOVA was used for multiple measurements.

Results

Relative Block of Reverse and Forward Mode of NCX Currents

We first compared the extent of reverse versus forward mode block during a voltage ramp protocol (Figure 1A). After

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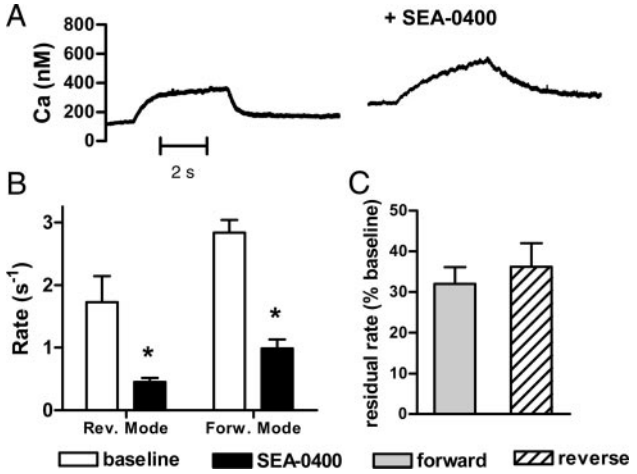


Figure 2. Block of Ca^{2+} fluxes through NCX by SEA-0400. A, $[Ca^{2+}]_i$ transients during 5-second depolarizing steps from −40 to +60 mV in the presence of nifedipine, ryanodine, and thapsigargin; Ca^{2+} influx and Ca^{2+} efflux rates were measured by exponential fitting. B, Mean rate constants of forward and reverse mode and inhibition by SEA-0400 (n=8). C, Residual fraction of the forward and reverse mode rate.

recording the baseline current, SEA-0400 was applied. A steady state was typically achieved after 5 minutes and the calculated difference current represented the SEA-sensitive current. Then, we added $NiCl_2$ to have full block of NCX and measured total I_{NCX}. From these data, the fractional block of I_{NCX} by SEA-0400 was calculated. At 1 μmol/L, SEA-0400 significantly decreased both the forward and reverse mode NCX. When measured at similar driving force of 60 mV, the extent of reverse mode block was larger than of forward mode (Figure 1B). This was more pronounced in mouse than in pig myocytes and, in both species, was more pronounced when pipette $[Na^+]$ was higher. When studying Ca^{2+} handling in the mouse, we used a lower dose of SEA-0400, 0.3 μmol/L, resulting in a similar block of forward mode I_{NCX} as in the pig (see below).

Relative Block of Ca^{2+} Influx via Reverse Mode and Efflux via Forward Mode

We next analyzed mode selectivity without Ca^{2+} buffering but with Ca^{2+} fluxes other than NCX inhibited (Ca^{2+} channels blocked with nifedipine, SR fluxes blocked with ryanodine and thapsigargin). In these conditions, a strong depolarizing pulse evokes reverse mode NCX and Ca^{2+} influx, and on repolarization, Ca^{2+} efflux occurs through forward mode NCX, illustrated in Figure 2A. The initial rates of Ca^{2+} increase on depolarization and of Ca^{2+} removal on repolarization were fit by a single exponential function (Figure 2B). The rate constants were decreased to a similar extent for forward and reverse mode (Figure 2C).

We also quantified the decrease of Ca^{2+} influx via reverse mode NCX in more physiological conditions, during short steps to +60 mV of 300 ms. The increase of $[Ca^{2+}]_i$ was reduced by 45±5% (n=10, data not shown).

Selectivity of NCX Block: Presence of Ca^{2+} Channel Block

Despite its higher selectivity for NCX than KBR-7943, SEA-0400 has also been reported to have some Ca^{2+} channel

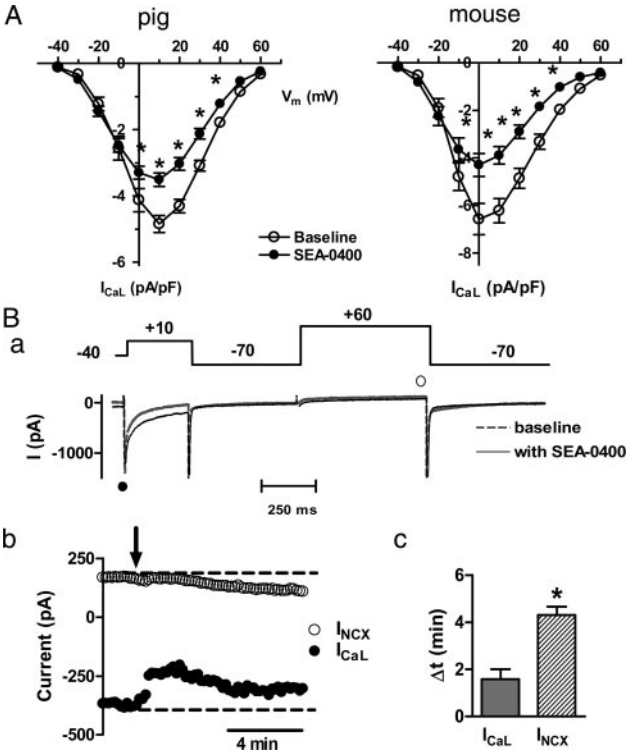


Figure 3. Block of I_{CaL} by SEA-0400. A, Current–voltage relation for peak inward I_{CaL} measured as the peak-minus-end of pulse current recorded with all K^+ currents blocked. For pig, we used 1 μmol/L SEA-0400 (n=20); for mouse, 0.3 μmol/L SEA-0400 (n=8). B, Time course of block of I_{CaL} and I_{NCX} in pig myocytes (a). A double-step protocol was repeated every 10 seconds during the wash in of 1 μmol/L SEA-0400 via a fast perfusion system (dashed trace at baseline, solid trace with SEA-0400; symbols indicate measurement of I_{CaL} and I_{NCX} shown in b). b, Peak inward I_{CaL} (solid symbols) and outward I_{NCX} (open symbols) at +60 mV during the wash in, started at arrow. In c, the average time to block is shown (n=11).

blocking effect. Current–voltage curves obtained before and after application of SEA-0400 (Figure 3A) suggested a decrease of I_{CaL} at +10 mV of 27% to 35%. Because of potentially confounding effects of rundown of I_{CaL}, we recorded simultaneously the time course of block of I_{NCX} and I_{CaL} during wash in of SEA-0400 (data obtained in pig myocytes). We used a double pulse protocol, repeated every 10 seconds, with a step to +10 mV to measure I_{CaL}, and a second depolarizing step to +60 mV to measure outward I_{NCX} (Figure 3Ba). These data confirmed that SEA-0400 at 1 μmol/L indeed reduced I_{CaL}. As described in dog myocytes,[16] the block of I_{CaL} had distinct properties. It occurred faster than the block of I_{NCX}, recovered partially, and was reversible in all cells that were tested (n=6), whereas the block of I_{NCX} by SEA-0400 on the same time scale was not. The difference in time course of the inhibition between I_{CaL} and I_{NCX} was highly significant (Figure 3Bb).

The block of I_{CaL} was also frequency-dependent and, in pig myocytes, increased from 35% at 0.1 Hz to 60% at 1 Hz ($P<0.05$, n=8). However, when measured in Na^+-free solutions and with intracellular Ca^{2+} buffering, current block and frequency dependence were blunted (29±5% at 0.1 Hz and 37±10% at 1 Hz; P=NS; n=5). This suggests that the block

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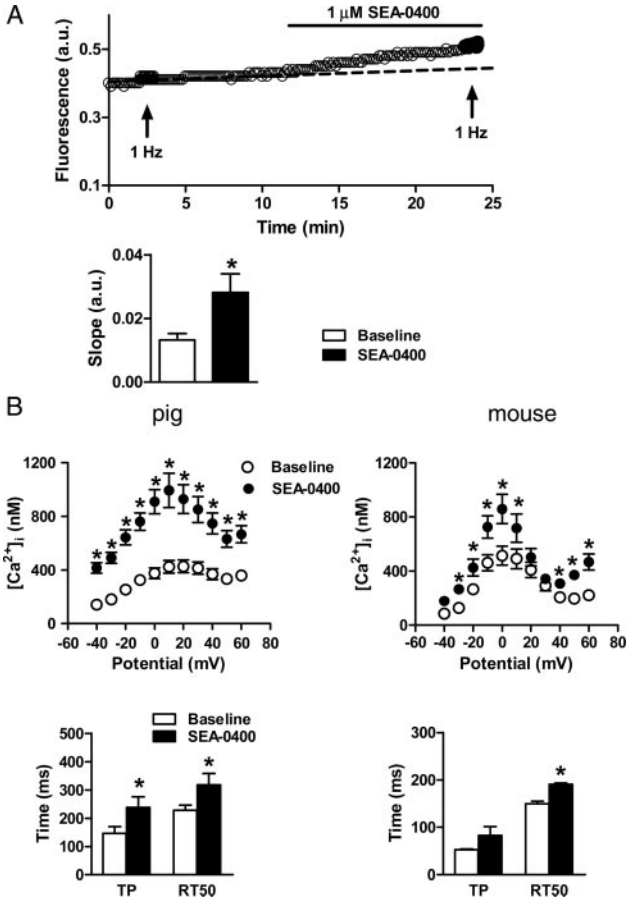


Figure 4. Increase in $[Ca^{2+}]_i$ with SEA-0400. A, Continuous record of baseline fluorescence before and during wash in of SEA-0400 in a pig myocyte; within the bar graph, the average data of the change in slope with SEA-0400 (n=9). B, Peak $[Ca^{2+}]_i$ during depolarizing steps and analysis of time to peak (TP) and half-time of relaxation (RT50) for the step to +10 mV (n=14 for pig, n=6 for mouse).

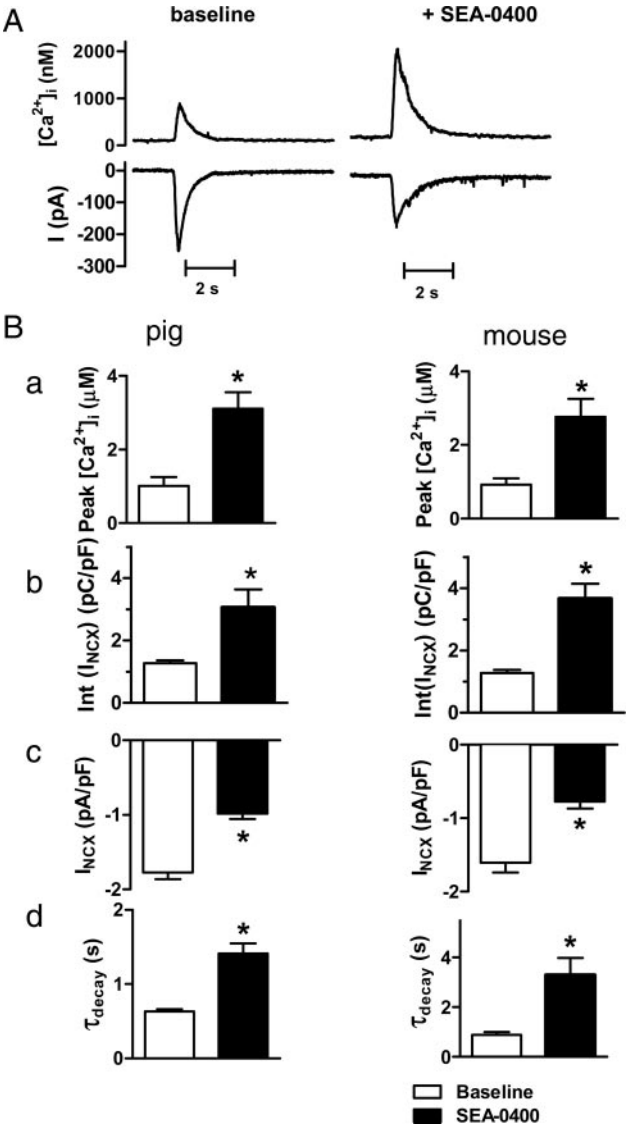


Figure 5. Increase in SR Ca^{2+} content with SEA-0400. A, Example of $[Ca^{2+}]_i$ and membrane currents during a 10-second application of 10 mmol/L caffeine before and after SEA-0400. B, Average data for peak $[Ca^{2+}]_i$ (a), integrated inward I_{NCX} (b), peak inward I_{NCX} (c), and, decay of the $[Ca^{2+}]_i$ transient (d) (n=9 for pig, n=6 for mouse).

of I_{CaL} is at least partially consequent on the effects of NCX block, most likely through the increase of resting $[Ca^{2+}]_i$.

SEA Increases $[Ca^{2+}]_i$

During wash in of SEA-0400 we observed an increase in both resting $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ transient amplitude. The effect on resting $[Ca^{2+}]_i$ was analyzed in a time course experiment designed to distinguish potentially confounding effects of alterations in dye loading from changes in resting $[Ca^{2+}]_i$ during long experiments (Figure 4A). After optimal dye loading, Ca^{2+} signals were recorded at 1 and 0.1 Hz for 1- and 10-minute periods in the absence of SEA-0400. The drug was then washed in during low frequency stimulation. The small increase in basal fluorescence during the first 12 minutes most likely reflects further dye loading and a small Ca^{2+} increase. However, after wash in of SEA-0400, there was a faster increase in the baseline values which must reflect an increase in resting $[Ca^{2+}]_i$. The slopes calculated by linear fit to the data points were significantly steeper after SEA-0400 application, implying a net increase in resting Ca^{2+} levels (Figure 4A, bar graph). This was also supported by analysis of resting cell length. Resting cell length, on average, decreased by 1.2±0.4% ($P<0.05$) in pig myocytes and by 1.3±0.4% ($P<0.05$) in mice.

The changes in $[Ca^{2+}]_i$ transient amplitude and the voltage dependence were evaluated using depolarizing steps to different voltages (Figure 4B). The peak Ca^{2+} values increased significantly with all voltage steps, both in pig and in mouse myocytes; resting $[Ca^{2+}]_i$ increased from 115±12 to 350±38 nmol/L in pig myocytes and from 67±11 to 124±20 nmol/L in mouse myocytes. For the step to +10 mV, we further analyzed the kinetics of the $[Ca^{2+}]_i$ transient. The time to peak and half-relaxation of the transients prolonged. The kinetics of I_{CaL} decline were not significantly altered (data not shown).

Effects of SEA-040 on the Sarcoplasmic Reticulum Ca^{2+} Load and Rate of Ca^{2+} Removal by NCX

We examined the Ca^{2+} load of the SR during caffeine-induced Ca^{2+} release (Figure 5A). Ca^{2+} load was increased as

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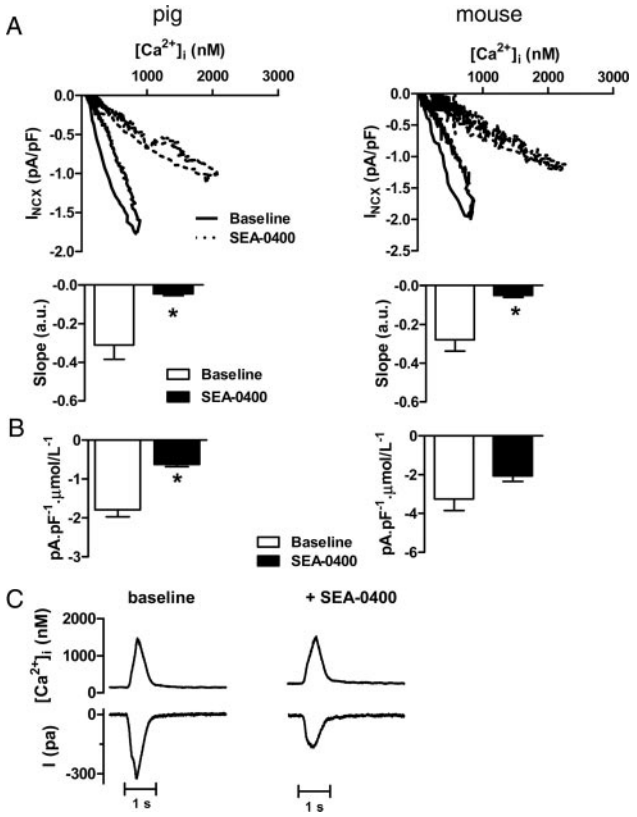


Figure 6. Reduced inward NCX currents with SEA-0400. A, Examples of phase-plane plots for I_{NCX} vs $[Ca^{2+}]_i$ before and after SEA-0400 with the mean data for the slope of the curve fitted to the decline of the $[Ca^{2+}]_i$ transient (n=9 pig and n=6 mouse). B, Current density of I_{NCX} normalized to $[Ca^{2+}]_i$, measured on repolarization from a step to +60 mV (n=14 pig and n=6 mouse). C, Example of $[Ca^{2+}]_i$ and membrane currents with a short (300 ms) application of 10 mmol/L caffeine before and after SEA-0400.

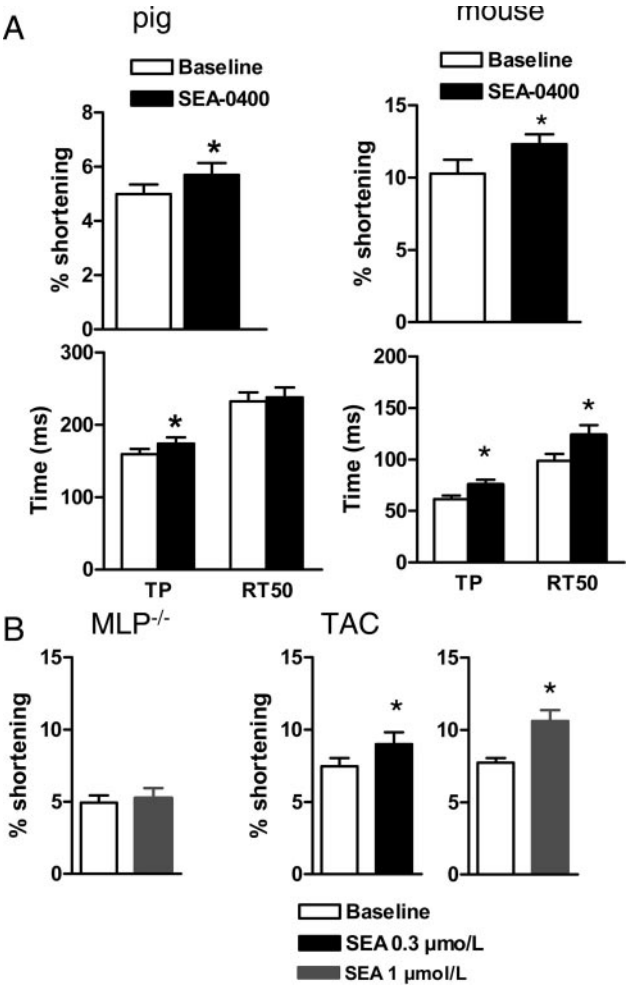


Figure 7. Inotropic effects of SEA-0400. A, Contraction amplitude and time to peak (TP) and to half-time to relaxation (RT50) of pig myocytes (n=18) and mouse myocytes (n=10); unloaded shortening during field stimulation at 1 Hz. B, Left, In myocytes from $MLP^{-/-}$ (n=10), shortening was reduced and did not increase with 1 μmol/L SEA-0400. Middle and Right, In myocytes from mice with TAC, shortening was also impaired, but the response to SEA was significant both at 0.3 μmol/L (n=8) and 1 μmol/L (n=12).

measured from the peak of the $[Ca^{2+}]_i$ transient as well as from the integrated I_{NCX} (Figure 5Ba and 5Bb), yet maximal peak inward I_{NCX} was reduced (Figure 5Bc).

From the caffeine experiments, we also quantified the rate of Ca^{2+} removal by NCX. The rate constant of Ca^{2+} decay was significantly increased (Figure 5Bd).

Extent of Block of the Inward I_{NCX}

We further measured the effectiveness of SEA-0400 on suppressing the inward current during SR Ca^{2+} release by quantifying I_{NCX} density as a function of $[Ca^{2+}]_i$ during the caffeine pulses (Figure 6A). In a phase plane analysis for the entire duration of the caffeine application, the loop shifted upward and to the right, and the slope became less steep after SEA-0400. The slope, a measure of current density as a function of $[Ca^{2+}]_i$ and calculated during the decline of the Ca^{2+} transient was reduced 3-fold for the pig and 2-fold for the mouse.

These data during caffeine release are consistent with measurements of inward I_{NCX} on repolarization from the step to +60 mV during the current–voltage protocol. Inward I_{NCX} density, normalized to $[Ca^{2+}]_i$ at that time, was likewise reduced 2- to 3-fold by SEA-0400 (Figure 6B).

The data of Figure 6B during 10s caffeine applications suggest that reduction of peak inward I_{NCX} may actually prolong I_{NCX}. We therefore also measured block of inward I_{NCX} during brief (300-ms) applications of caffeine in pig myocytes. This protocol mimics more closely spontaneous Ca^{2+} release as it allows normal SR reuptake (Figure 6C).[29] Under those conditions peak inward I_{NCX} was similarly reduced (from −1.85 to −0.61 pA/pF per μmol/L Ca; n=5) but without prolongation (τ from 235 to 279 ms).

SEA-0400 As Inotropic Agent

We measured the effects of SEA-0400 on the amplitude and kinetics of contraction during field stimulation at 1 Hz (Figure 7). In pig myocytes, the amplitude of shortening increased; the time to peak shortening also increased with a trend to slower relaxation, but this was not significant. SEA-0400 never induced spontaneous activity (18 cells tested). In mouse myocytes, contraction amplitude similarly

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increased ($P=0.07$) and the kinetics were slower, both for rate of contraction and relaxation.

To see whether the positive inotropic effect could be advantageous in heart failure, we tested the effects of SEA-0400 in $MLP^{-/-}$ mice, a known model for heart failure (Figure 7B, left). Amplitude of contraction was less than in WT at baseline, but, surprisingly, SEA-0400 even at 1 μmol/L failed to increase the amplitude, though relaxation was slowed (half-time to relaxation, from 121±5 to 140±13 ms) and resting length decreased by 2.3±0.9%. We tested the same concentration in WT myocytes, in which a significant increase was seen (not shown), with occurrence of spontaneous activity in 7 of 8 cells. These data suggested that, in the $MLP^{-/-}$ myocytes, the SR content failed to increase. Indeed, the amplitude of $[Ca^{2+}]_i$ transients did not increase and neither did the amplitude of the caffeine-induced Ca^{2+} release or the integrated I_{NCX} (data not shown). There was however an increase in resting $[Ca^{2+}]_i$. Presence of block of I_{NCX} was evidenced by the decrease of peak inward current decreased (to −0.50±0.07 versus −1.42±0.19 pA/pF at baseline, $P<0.01$) and the rate of decay of the caffeine-induced Ca^{2+} transient (τ, 1.89±0.30 versus 0.96±0.12 seconds at baseline, $P<0.01$). We also tested the effect of SEA-0400 in myocytes from mice with 8 weeks of transverse aortic constriction (TAC), another well-established model of cardiac hypertrophy and failure. Heart weight/body weight ratio was, on average, increased by 44%, and lung weight by 48% ($n_{mice}=5$). Contraction of isolated myocytes was less impaired than in the $MLP^{-/-}$ mice, and SEA-0400 had a significant inotropic effect both at 0.3 and 1 μmol/L (Figure 7B, middle and right).

Discussion

Selectivity and Mode Dependence of NCX Block by SEA-0400

Earlier studies in guinea pig had reported a very high selectivity of SEA-0400 with only a 10% block of I_{CaL} at the EC50 of 50 nmol/L.[22] In the dog, a concentration of 0.3 μmol/L, close to the EC50 for the inward I_{NCX}, reduced I_{CaL} by 10%, and at 1 μmol/L, by 20%.[26] SEA-0400 has been used at 1 and 10 μmol/L in the mouse,[30] but its selectivity was not previously determined. In the present study, we chose to work with a concentration of 1 μmol/L, a concentration that has been used in most functional studies so far. In the pig, this resulted in a block of I_{CaL} of some 25%, whereas it was even higher in the mouse (data not shown). We also measured I_{CaL} in the mouse with a lower dose of 0.3 μmol/L, but block was still ≈25%. These data suggest that if the goal is a block of I_{NCX} of at least 50%, concomitant block of I_{CaL} is unavoidable. They also indicate that beneficial effects of SEA-0400 cannot be equivocally ascribed to NCX block, but could be related to the concomitant I_{CaL} block.

Possible mode dependence of NCX block, ie, different degree of block in reverse versus forward mode, remains an issue of debate. It was first described for KB-R7943,[31] followed by studies that found a much less pronounced, or even absent, mode dependence.[21,22] In dog myocytes, KB-R7943 showed mode dependence even during symmetrical ramp protocols.[26] For SEA-0400, giant-patch experiments showed higher potency of the drug when the reverse mode was favored by very high cytosolic Na^+ concentration.[14] These observations led to the idea that drug-binding could be mode-dependent and favored by the Na-dependent inactivated state. In our experiments, we recorded simultaneously forward and reverse mode and measured currents at equal distance from the reversal potential. We observed only a small but significant difference, with reverse mode blocked to a larger extent, in particular in mouse myocytes. On the other hand, increasing the pipette Na concentration did not greatly affect the degree of block. These results are not easily reconciled with the Na-dependent hypothesis, unless Na binding would be voltage-dependent. We also measured rates of Ca^{2+} influx and removal by NCX and could not demonstrate a mode dependence of block (Figure 2).

We further studied NCX block under more physiological conditions. When normalized to $[Ca^{2+}]_i$, inward I_{NCX} density was reduced more than 3-fold during SR Ca^{2+} release. The suppression of outward current during the double pulse protocol amounted to approximately 50% (data not shown), consistent with the reduction of Ca^{2+} influx via reverse mode by ≈50%.

These data suggest that mode dependence is absent or even reversed during normal excitation–contraction coupling. Possibly, block of inward I_{NCX} is potentiated by high $[Ca^{2+}]_i$ because the highest degree of block was seen at the peak of caffeine-induced Ca^{2+} release (82% reduction of I_{NCX}; Figure 6A).

Ca^{2+} Handling With SEA-0400

In both mouse and pig myocytes, SEA-0400 increased the amplitude of the Ca^{2+} transients, increased resting Ca^{2+}, and increased the Ca^{2+} content of the SR. The rate of decline of the Ca^{2+} transient was slowed down. Although a number of studies have used SEA-0400 as a tool to block NCX, reports on the consequences for Ca^{2+} handling are limited. In the mouse, the increase of $[Ca^{2+}]_i$ transient amplitude was reported before[30] but with 10 μmol/L SEA-0400. These authors observed, but did not further comment on, the increase in resting Ca^{2+}. They also did not report an increase of spontaneous activity, but it is possible that the higher dose of SEA-0400 had a higher concomitant degree of I_{CaL} block; this was not tested. In the dog, only a minor effect on $[Ca^{2+}]_i$ transients was seen, possibly because of the block of I_{CaL}.[26]

The effects on Ca^{2+} handling are consistent with a net decrease in Ca^{2+} removal and gain of Ca^{2+} load of the myocytes in mice and pig, despite moderate block of I_{CaL}. In the NCX knockout mouse, there was no such net gain of Ca^{2+} and this was ascribed to the reduction of I_{CaL} by 60%,[19] which exceeds the reduction with SEA-0400. It is therefore conceivable that drugs that would have a higher degree of I_{CaL} block would not result in cellular Ca^{2+} gain.

Our data indicate clearly that during normal excitation–contraction coupling, the physiology of NCX with net Ca^{2+} removal during a cardiac cycle outweighs an intrinsic mode dependence of block. Beneficial effects of NCX block or knockout on ischemia-reperfusion injury could result from a predominance of Ca^{2+} influx via NCX under these conditions but at the same time suggest there are alternate ways for Ca^{2+} removal. Another possible interpretation is that the mode

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dependence is much more pronounced under those conditions because of very high $[Na^+]$. Our current data with 20 versus 10 mmol/L pipette Na^+ do not reveal such big differences but may underestimate the increase in subsarcolemmal Na^+ during ischemia/reperfusion.

Species Differences in NCX Inhibition

When we set up the study, we expected to find significant differences between mouse and pig myocytes. In the mouse, Ca^{2+} reuptake into the SR is the major Ca^{2+} removal system, and, based on the data from the NCX knockout mouse, we expected less effect on Ca^{2+} handling. We also hypothesized that mouse myocytes would have higher $[Na^+]_i$ and more preferential reverse mode inhibition, which would result in a reduction of contraction rather than increase.

The baseline characteristics in kinetics of $[Ca^{2+}]_i$ transients and contractions are striking, with the mouse being faster and having larger contractions (Figures 4 and 7). Differences in the effect of NCX inhibition, however, are small. Even though the mode dependence in the ramp protocol at 1 μmol/L SEA-0400 was slightly more pronounced, the predominant effect in mice of NCX inhibition was still a gain of Ca^{2+}. During field stimulation, mouse myocytes had a tendency to spontaneous activity after several minutes of exposure to 1 μmol/L SEA-0400, which was never seen in pig myocytes. This could correspond to a higher potential for SR accumulation and there was indeed a trend toward higher increase of SR content (Figure 5).

Overall, our data suggest that quantitative differences are present but do not indicate differences in the principal actions of NCX inhibition between mouse or pig myocytes.

NCX Block As an Positive Inotropic Intervention

In healthy pig and mouse myocytes, SEA-0400 increased amplitude of contractions, consistent with the increase in $[Ca^{2+}]_i$. In myocytes from dogs with heart failure, NCX block with intracellular XIP not only increased the amplitude of the Ca^{2+} transient but also led to a faster relaxation.[20] This was ascribed to a shift from excessive NCX removal to enhanced SR uptake. In the $MLP^{-/-}$ mouse with heart failure, we could not achieve such beneficial effects. We have previously reported that, in these mice, the SR loading is not diminished at baseline but the SR has a reduced capacity for increasing its load.[24] The ryanodine receptor also has a higher degree of phosphorylation, and myocytes may have an increased cycling across the sarcolemma through NCX.[24] For these reasons, NCX block may be unable to enhance SR Ca^{2+} uptake and raise SR Ca^{2+} load in this heart failure model. In contrast, in myocytes from mice with TAC, the inotropic effect was preserved. These observations indicate that the effects in heart failure depend on the associated changes in other transports or myofilament function. The value of SEA-0400 in heart failure clearly needs to be studied further and cannot be extrapolated simply from data obtained in healthy myocytes. Large animal models with a greater reliance on NCX in heart failure should be included because their response may be more pronounced.

Perspectives for NCX Inhibitors

SEA-0400 was very effective in reducing the maximal peak current associated with Ca^{2+} release at resting membrane potentials, as measured during caffeine-induced Ca^{2+} release. In the presence of a functional SR, this could provide specific antiarrhythmic protection, currently not achieved with any other class of drugs. Several groups have reported reduction of delayed afterdepolarizations and also early afterdepolarizations using either KBR-7943 or SEA-0400. Given that none of these studies used concentrations that were selective for NCX, concomitant Ca^{2+} channel block may have contributed to the beneficial effects. To further tease out the role of NCX inhibition proper, a more selective blocker will be useful. Yet, in the clinical perspective, a concomitant block of I_{CaL} may be an advantage and part of the therapeutic profile of antiarrhythmic drugs.[11,16,32]

Our data strongly suggest that NCX block can have adverse effects in heart failure, because it raises resting Ca^{2+} and slows down relaxation. Yet, there is also a potential benefit from increasing SR Ca^{2+} load. This requires that SR uptake is preserved, also to prevent an excessive raise in resting Ca^{2+}.

In conclusion, further development of NCX inhibitors should weigh the potential benefits of current block against the effects on Ca^{2+} handling. Inhibitors of NCX without effects on other Ca^{2+} transport are highly desirable for research purposes but may not necessarily be the first choice for clinical use. If mode selectivity of NCX inhibition could be developed, it would have clear advantages, but this goal has not yet been achieved.

Acknowledgments

SEA-0400 was generously supplied by Dr Fülöp (University of Szeged). We thank E. Detre, C. Huysmans, and K. Vermeulen for assistance.

Sources of Funding

This study was supported by grants to K.R.S from the Fund for Scientific Research (Flanders) (grant G.0384.07), the European Union (grant LSHM-CT-2005-018833, EUGeneHeart), and the Belgian Science Program (grant IAP6/31).

Disclosures

None.

References

1. Eisner DA, Choi HS, Diaz ME, O'Neill SC, Trafford AW. Integrative analysis of calcium cycling in cardiac muscle. *Circ Res*. 2000;87:1087–1094.
2. Bers DM. Cardiac excitation-contraction coupling. *Nature*. 2002;415:198–205.
3. Egger M, Niggli E. Regulatory function of Na-Ca exchange in the heart: milestones and outlook. *J Membr Biol*. 1999;168:107–130.
4. Sipido KR, Bito V, Antoons G, Volders PG, Vos MA. Na/Ca exchange and cardiac ventricular arrhythmias. *Ann N Y Acad Sci*. 2007;1099:339–348.
5. Jiang D, Wang R, Xiao B, Kong H, Hunt DJ, Choi P, Zhang L, Chen SR. Enhanced store overload-induced Ca2+ release and channel sensitivity to luminal Ca2+ activation are common defects of RyR2 mutations linked to ventricular tachycardia and sudden death. *Circ Res*. 2005;97:1173–1181.
6. Liu N, Colombi B, Memmi M, Zissimopoulos S, Rizzi N, Negri S, Imbriani M, Napolitano C, Lai FA, Priori SG. Arrhythmogenesis in

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catecholaminergic polymorphic ventricular tachycardia: insights from a RyR2 R4496C knock-in mouse model. *Circ Res*. 2006;99:292–298.
7. Venetucci LA, Trafford AW, Eisner DA. Increasing ryanodine receptor open probability alone does not produce arrhythmogenic calcium waves: threshold sarcoplasmic reticulum calcium content is required. *Circ Res*. 2007;100:105–111.
8. Pieske B, Maier LS, Piacentino V, Weisser J, Hasenfuss G, Houser S. Rate dependence of [Na+]i and contractility in nonfailing and failing human myocardium. *Circulation*. 2002;106:447–453.
9. Pogwizd SM. Clinical potential of sodium-calcium exchanger inhibitors as antiarrhythmic agents. *Drugs*. 2003;63:439–452.
10. Hobai IA, O'Rourke B. The potential of Na^+/Ca^{2+} exchange blockers in the treatment of cardiac disease. *Expert Opin Investig Drugs*. 2004;13:653–664.
11. Sipido KR, Varro A, Eisner D. Sodium calcium exchange as a target for antiarrhythmic therapy. *Handb Exp Pharmacol*. 2006;(171):159–199.
12. Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem*. 1996;271:22391–22397.
13. Shigekawa M, Iwamoto T. Cardiac Na(+)-Ca(2+) exchange: molecular and pharmacological aspects. *Circ Res*. 2001;88:864–876.
14. Lee C, Visen NS, Dhalla NS, Le HD, Isaac M, Choptiany P, Gross G, Omelchenko A, Matsuda T, Baba A, Takahashi K, Hnatowich M, Hryshko LV. Inhibitory profile of SEA0400 [2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline] assessed on the cardiac Na+-Ca2+ exchanger, NCX1.1. *J Pharmacol Exp Ther*. 2004;311:748–757.
15. Satoh H, Ginsburg KS, Qing K, Terada H, Hayashi H, Bers DM. KB-R7943 block of Ca^{2+} influx via Na^+/Ca^{2+} exchange does not alter twitches or glycoside inotropy but prevents Ca^{2+} overload in rat ventricular myocytes. *Circulation*. 2000;101:1441–1446.
16. Nagy ZA, Virag L, Toth A, Biliczki P, Acsai K, Banyasz T, Nanasi P, Papp JG, Varro A. Selective inhibition of sodium-calcium exchanger by SEA-0400 decreases early and delayed afterdepolarization in canine heart. *Br J Pharmacol*. 2004;143:827–831.
17. Imahashi K, Pott C, Goldhaber JI, Steenbergen C, Philipson KD, Murphy E. Cardiac-specific ablation of the Na^+-Ca^{2+} exchanger confers protection against ischemia/reperfusion injury. *Circ Res*. 2005;97:916–921.
18. Henderson SA, Goldhaber JI, So JM, Han T, Motter C, Ngo A, Chantawansri C, Ritter MR, Friedlander M, Nicoll DA, Frank JS, Jordan MC, Roos KP, Ross RS, Philipson KD. Functional adult myocardium in the absence of Na^+-Ca^{2+} exchange: cardiac-specific knockout of NCX1. *Circ Res*. 2004;95:604–611.
19. Pott C, Philipson KD, Goldhaber JI. Excitation-contraction coupling in Na^+-Ca^{2+} exchanger knockout mice: reduced transsarcolemmal Ca^{2+} flux. *Circ Res*. 2005;97:1288–1295.
20. Hobai IA, Maack C, O'Rourke B. Partial inhibition of sodium/calcium exchange restores cellular calcium handling in canine heart failure. *Circ Res*. 2004;95:292–299.
21. Kimura J, Watano T, Kawahara M, Sakai E, Yatabe J. Direction-independent block of bi-directional Na^+/Ca^{2+} exchange current by KB-R7943 in guinea-pig cardiac myocytes. *Br J Pharmacol*. 1999;128:969–974.
22. Tanaka H, Nishimaru K, Aikawa T, Hirayama W, Tanaka Y, Shigenobu K. Effect of SEA0400, a novel inhibitor of sodium-calcium exchanger, on myocardial ionic currents. *Br J Pharmacol*. 2002;135:1096–1100.
23. Stankovicova T, Szilard M, De Scheerder I, Sipido KR. M cells and transmural heterogeneity of action potential configuration in myocytes from the left ventricular wall of the pig heart. *Cardiovasc Res*. 2000;45:952–960.
24. Antoons G, Vangheluwe P, Volders PGA, Bito V, Holemans P, Ceci M, Wuytack F, Caroni P, Mubagwa K, Sipido KR. Increased phospholamban phosphorylation limits the force-frequency response in the MLP−/− mouse with heart failure. *J Mol Cell Cardiol*. 2006;40:350–360.
25. Arber S, Hunter JJ, Ross JJ, Hongo M, Sansig G, Borg J, Pierrard JC, Chien KR, Caroni P. MLP-deficient mice exhibit a disruption of cardiac architectural organization, dilated cardiomyopathy, and heart failure. *Cell*. 1997;88:393–403.
26. Birinyi P, Acsai K, Banyasz T, Toth A, Horvath B, Virag L, Szentandrassy N, Magyar J, Varro A, Fülöp F, Nanasi PP. Effects of SEA0400 and KB-R7943 on Na^+/Ca^{2+} exchange current and L-type Ca^{2+} current in canine ventricular cardiomyocytes. *Naunyn Schmiedebergs Arch Pharmacol*. 2005;372:63–70.
27. Antoons G, Mubagwa K, Nevelsteen I, Sipido KR. Mechanisms underlying the frequency dependence of contraction and $[Ca^{2+}]_i$ transients in mouse ventricular myocytes. *J Physiol (Lond)*. 2002;543:889–898.
28. Trafford AW, Diaz ME, Eisner DA. A novel, rapid and reversible method to measure Ca buffering and time-course of total sarcoplasmic reticulum Ca content in cardiac ventricular myocytes. *Pflugers Arch*. 1999;437:501–503.
29. Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure: roles of sodium-calcium exchange, inward rectifier potassium current, and residual β-adrenergic responsiveness. *Circ Res*. 2001;88:1159–1167.
30. Tanaka H, Namekata I, Takeda K, Kazama A, Shimizu Y, Moriwaki R, Hirayama W, Sato A, Kawanishi T, Shigenobu K. Unique excitation-contraction characteristics of mouse myocardium as revealed by SEA0400, a specific inhibitor of Na^+-Ca^{2+} exchanger. *Naunyn Schmiedebergs Arch Pharmacol*. 2005;371:526–534.
31. Watano T, Kimura J, Morita T, Nakanishi H. A novel antagonist, No. 7943, of the Na^+/Ca^{2+} exchange current in guinea-pig cardiac ventricular cells. *Br J Pharmacol*. 1996;119:555–563.
32. Bito V, Dauwe D, Verdonck F, Mubagwa K, Sipido KR. The amiodarone derivative KB130015 [2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl) benzofuran] induces a Na^+-dependent increase of $[Ca^{2+}]$ in ventricular myocytes. *J Pharmacol Exp Ther*. 2006;316:162–168.

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