Differing cardioprotective efficacy of the Na\(^+\)/Ca\(^{2+}\) exchanger inhibitors SEA0400 and KB-R7943

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Magee, William P., Gayatri Desmukh, Michael P. DeNinno, Jill C. Sutt, Justin G. Chapman, and W. Ross Tracey. Differing cardioprotective efficacy of the Na\(^+\)/Ca\(^{2+}\) exchanger inhibitors SEA0400 and KB-R7943. Am J Physiol Heart Circ Physiol 284: H903–H910, 2003. First published November 21, 2002; 10.1152/ajpheart.00784.2002.—KB-R7943 and SEA0400 are Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) inhibitors with differing potency and selectivity. The cardioprotective efficacy of these NCX inhibitors was examined in isolated rabbit hearts (Langendorff perfused) subjected to regional ischemia (coronary artery ligation) and reperfusion. KB-R7943 and SEA0400 elicited concentration-dependent reductions in infarct size (SEA0400 EC\(_{50}\); 5.7 nM). SEA0400 was more efficacious than KB-R7943 (reduction in infarct size at 1 \(\mu\)M: SEA0400, 75%; KB-R7943, 40%). Treatment with either inhibitor yielded similar reductions in infarct size whether administered before or after regional ischemia. SEA0400 (1 \(\mu\)M) improved postischemic recovery of function (\(\pm dP/dt\)), whereas KB-R7943 impaired cardiac function at \(\pm 1\) \(\mu\)M. At 5–20 \(\mu\)M, KBR-7943 elicited rapid and profound depressions of heart rate, left ventricular developed pressure, and \(\pm dP/dt\). Thus the ability of KB-R7943 to provide cardioprotection is modest and limited by negative effects on cardiac function, whereas the more selective NCX inhibitor SEA0400 elicits marked reductions in myocardial ischemic injury and improved \(\pm dP/dt\). NCX inhibition represents an attractive approach for achieving clinical cardioprotection.

ischemia; reperfusion; heart; infarct; rabbit

THE Na\(^+\)/Ca\(^{2+}\) EXCHANGER (NCX) plays an important role in regulating cardiomyocyte Ca\(^{2+}\) homeostasis. NCX1 is the predominant isoform present in the heart, whereas NCX2 and NCX3 are primarily restricted to the brain and skeletal muscle (17, 21, 22). Within the cardiomyocyte, sarcomemmal NCX1 is the major route of Ca\(^{2+}\) extrusion from the cytosol, responsible for ~20–25% of the reduction in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) during diastole (the remainder being due to Ca\(^{2+}\) sequestration by sarcoplasmic Ca\(^{2+}\)-ATPase) (for reviews, see Refs. 1–5). During removal of Ca\(^{2+}\) from the cytosol, NCX operates in the “forward” mode and exchanges Na\(^+\) for Ca\(^{2+}\) with a 3:1 stoichiometry (for a review, see Ref. 4). However, this exchanger is bidirectional, being tightly regulated by membrane potential and transmembrane gradients of Na\(^+\) and Ca\(^{2+}\). Changes in ionic conditions, such as those observed during ischemia [i.e., elevated intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\))] will cause NCX to function in the “reverse” mode, resulting in an increase in [Ca\(^{2+}\)]\(_i\); not surprisingly, NCX has been implicated in the development of myocardial ischemic injury (6, 9, 20, 24, 25, 31).

Given the potential clinical benefit of inhibiting NCX activity during myocardial ischemia-reperfusion, pharmacological approaches have been pursued to both inhibit and clarify the importance of this exchanger. However, a dearth of selective NCX inhibitors has impeded progress until the recent identification of KB-R7943 and SEA0400. KB-R7943 is reported to have a modest NCX selectivity (20- to 40-fold) versus other ion channels (e.g., voltage-gated Na\(^+\) current, Ca\(^{2+}\) current, and inward rectifier K\(^+\) current) and to be selective for the reverse mode (50-fold vs. the forward mode) of NCX (10, 11, 28). SEA0400 possesses a higher NCX potency and greater selectivity than KB-R7943 against the L-type Ca\(^{2+}\) channel and several receptors/enzymes, although it lacks selectivity for the reverse versus forward mode of the exchanger (18). Whereas KB-R7943 has been demonstrated to reduce some markers of cardiac ischemia-reperfusion injury (arrhythmias, hypercontracture, and enzyme release) (16, 19), its ability to reduce infarct size in the whole heart has not been carefully evaluated. SEA0400 reduced infarct volume in a model of middle cerebral artery occlusion (18), but its efficacy at preventing cardiac ischemia-reperfusion injury is unknown. Thus we investigated the effects of KB-R7943 and SEA0400 on infarct size and cardiac function in the ischemic-reperfused heart to establish the relative efficacy of these agents and to better understand the contribution of NCX activation to myocardial ischemic injury.

MATERIALS AND METHODS

This investigation conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

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In vitro (Langendorff) preparation. Male New Zealand White rabbits (3–4 kg, Covance; Denver, PA) were anesthetized by an intravenous administration of pentobarbital sodium (30 mg/kg), followed by intubation and ventilation with 100% O2 using a positive pressure ventilator. A left thoracotomy was performed, the heart was exposed, and a snare (2-0 silk) was placed loosely around a prominent branch of the left coronary artery. The heart was rapidly removed from the chest, mounted on a Langendorff apparatus, and maintained by retrograde perfusion (nonrecirculating) with a modified Krebs solution composed of (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 24.8 NaHCO3, 2.5 CaCl2, and 10 glucose) at a constant pressure of 80 mmHg and temperature of 38.5°C. Perfusion pH was maintained at 7.4–7.5 by bubbling with 95% O2-5% CO2. The temperature of the heart was 38.5°C. The heart was not used unless the heart rate fell below 180 beats/min above were not observed, the heart was discarded. Pacing was not used unless the heart rate fell below 180 beats/min before the 30-min period of regional ischemia; in this case, the heart was paced at 200 beats/min, which was the average spontaneous rate observed.

**Experimental protocols.** Thirty minutes of regional ischemia was produced by tightening the snare around the branch of the coronary artery. At the end of this period, the snare was released, and the heart was reperfused for an additional 120 min. KB-R7943 or SEA0400 perfusion was started either 30 min before the regional ischemia or 1 min before reperfusion and was continued for the duration of the experiment (120 min of reperfusion). Control hearts did not receive any drug. In some experiments, the effects of constant perfusion of KB-R7943 on cardiac function and CF were evaluated in the absence of regional ischemia and reperfusion.

**Determination of infarct size.** After completion of each experiment, the coronary artery snare was retightened, and a 0.5% suspension of fluorescent zinc cadmium sulfide particles (1–10 μm) was perfused through the heart to delineate the area at risk (AAR; nonlabeled) in the LV for infarct development. The heart was removed from the Langendorff apparatus, blotted dry, weighed, wrapped in aluminum foil, and stored overnight at −20°C. Frozen hearts were sliced into 2-mm transverse sections and incubated with 1% triphenyltetrazolium chloride in phosphate-buffered saline for 20 min at 37°C to delineate noninfarcted (stained) from infarcted (nonstained) LV tissue. The infarct area (IA) and AAR were calculated for each slice of the LV using video-captured images and ETC3000 image-analysis software (Engineering Technology Center; Mystic, CT), followed by addition of the values for each tissue slice to obtain the total IA and total AAR for each heart. To normalize the IA for differences in the AAR between hearts, the infarct size was expressed as the ratio of the IA versus AAR (%IA/AAR).

**Western blots.** To determine whether ischemia-reperfusion changed myoccardial NCX1 protein expression, tissue samples (right ventricular free wall, LV AAR) from hearts exposed to either 30 min of ischemia and 120 min of reperfusion or control hearts (temporally matched) were suspended in 10 mM Tris buffer (pH 7.5) containing 300 mM sucrose, 1 mM dithiothreitol, and protease inhibitors (Complete, Boehringer Mannheim; Indianapolis, IN) and homogenized. Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical; Rockford, IL) with BSA as a stan-

**Table 1. Cardiac function and coronary flow data from isolated rabbit hearts**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HR CF LVDP</th>
<th>HR CF LVDP</th>
<th>HR CF LVDP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>198 ± 10</td>
<td>6.0 ± 0.3</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>KB-R7943</td>
<td>5</td>
<td>211 ± 9</td>
<td>5.9 ± 0.3</td>
<td>86 ± 4</td>
</tr>
<tr>
<td>0.3 μM</td>
<td>5</td>
<td>203 ± 6</td>
<td>5.6 ± 0.1</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>5</td>
<td>203 ± 5</td>
<td>5.5 ± 0.2</td>
<td>79 ± 5</td>
</tr>
<tr>
<td>SEA0400</td>
<td>5</td>
<td>204 ± 7</td>
<td>5.6 ± 0.3</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>0.001 μM</td>
<td>5</td>
<td>198 ± 9</td>
<td>5.3 ± 0.3</td>
<td>101 ± 5</td>
</tr>
<tr>
<td>0.01 μM</td>
<td>5</td>
<td>201 ± 6</td>
<td>5.3 ± 0.1</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>0.1 μM</td>
<td>5</td>
<td>206 ± 6</td>
<td>5.7 ± 0.4</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>1.0 μM</td>
<td>5</td>
<td>213 ± 6</td>
<td>6.2 ± 0.3</td>
<td>100 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rabbit hearts. HR, heart rate (in beats/min); CF, total coronary flow rate (in ml·min⁻¹·g⁻¹); LVDP, left ventricular developed pressure (in mmHg). *P < 0.05 vs. preischemic values; †P < 0.05 vs. control values.

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Twenty micrograms of protein were separated by 10\% SDS-PAGE and transferred to a membrane. The membrane was blocked with 5\% nonfat milk for 1 h at room temperature and then probed overnight with either NCX1 (mouse monoclonal, 1:1,000 dilution, Chemicon International; Temecula, CA) or GAPDH (mouse monoclonal, 1:1,000 dilution, Research Diagnostics; Flanders, NJ) antibodies in blocking solution at 4°C. After being washed, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) and visualized with enhanced chemiluminescence (ECL-Plus, Amersham Pharmacia Biotech; Piscataway, NJ). ECL films were scanned and quantitated by densitometry; NCX1 expression was normalized to GAPDH.

**Data expression and analysis.** Data are expressed as means ± SE. ANOVA was used for between-group comparisons of the AAR expressed as a percentage of LV areas (%AAR/LV), and for between-group comparisons of cardiac function/CF parameters. Comparison of cardiac function/CF parameters within groups (before and after coronary artery occlusion) and Western blot densitometry was performed by *t*-test. %IA/AAR values were compared using a Mann-Whit.
ney test. A Bonferroni correction was applied to multiple t-test or Mann-Whitney comparisons. A P value of <0.05 was considered statistically significant.

Drugs and drug preparation. KB-R7943 (11) and SEA0400 (18) were synthesized at Pfizer Global Research and Development (Groton, CT) (Fig. 1). Drugs were dissolved in DMSO and diluted in buffer; the final DMSO concentration was <0.1%, which had no effect on infarct size (27).

RESULTS

In the Langendorff-perfused hearts, LVDP and CF were significantly (P < 0.05) reduced in all groups by occlusion of the coronary artery, confirming that ischemia was achieved (Table 1). The %AAR/LV was 33 ± 3% (n = 5) for the control group; %AAR/LV values for the KB-R7943- and SEA0400-treated groups were not significantly different from the control group.

SEA0400, when administered before the regional ischemia, produced a significant (P < 0.05) concentration-dependent reduction in infarct size (Fig. 2A). The maximum reduction in infarct size (75%) was achieved at 0.1 μM SEA0400 (control: 55 ± 3% IA/AAR; 0.1 μM SEA0400: 13 ± 1% IA/AAR), and the EC50 was 5.7 nM (Fig. 2A). Similarly, KB-R7943 elicited a significant (P < 0.05) concentration-dependent reduction in myocardial ischemic injury (Fig. 2B), although a full concentration-response curve could not be obtained because of deleterious effects on cardiac function (described below). Infarct size was reduced by 40% at a KB-R7943 concentration of 1 μM (control: 55 ± 3% IA/AAR; 1 μM KB-R7943: 33 ± 1% IA/AAR). In addition, the extent of cardioprotection provided by both KB-R7943 (1 μM) and SEA0400 (1 μM) when administered 1 min before reperfusion was similar to that produced by either agent when given before the regional ischemia (Fig. 2).

The effects of SEA0400 and KB-R7943 on cardiac function were markedly different. At 1 μM, neither SEA0400 nor KB-R7943 demonstrated any direct im-

Fig. 3. Effect of SEA0400 (1 μM) and KB-R7943 (1 μM) on functional recovery [+dP/dt (A) and −dP/dt (B)] in the isolated rabbit heart. SEA0400 or KB-R7943 were constantly perfused through the heart beginning 30 min before the regional ischemia, as described in MATERIALS AND METHODS. Data are means ± SE and were sampled at 1-min intervals for each group; n = 5 for each group. The response of the SEA0400-treated hearts was significantly different (P < 0.05) versus controls, whereas the KB-R7943-treated group was not statistically different from controls.
pairment of cardiac function before the ischemic period (Table 1). However, 1 μM KB-R7943-treated hearts appeared to have depressed LVDP at the end of the ischemic period, which became significantly \( P < 0.05 \) reduced by 35% versus control at the end of the reperfusion period (Table 1). Recovery of function \( (\pm dP/dt) \) of hearts treated with 1 μM KB-R7943 also tended to be impaired (Fig. 3), although these changes were not statistically significant \( P > 0.05 \). In contrast, the same concentration of SEA0400 (1 μM) did not adversely affect LVDP postischemia (Table 1) and elicited a marked and significant \( P < 0.05 \) improvement in \( \pm dP/dt \) (Fig. 3). Administration of KB-R7943 at concentrations >1 μM resulted in profound deleterious changes in cardiac function and coronary flow (Fig. 4); 5 μM KB-R7943 \( (n = 4) \) decreased heart rate, LV systolic pressure, and \( \pm dP/dt \); LV diastolic pressure increased during this time, leading to a reduction in LVDP. Coronary flow initially increased immediately after KB-R7943 administration, followed by a gradual decline. Exploratory studies with KB-R7943 at 10 and 20 μM \( (n = 2 \text{ for each group; data not shown}) \) demonstrated changes in cardiac function and coronary flow similar to those observed at the 5 μM concentration. Because of these rapid changes in cardiac function/coronary flow and the ultimate failure of the hearts, the effects of KB-R7943 on infarct size at concentrations >1 μM could not be examined.

Myocardial NCX1 protein expression and the effect of ischemia-reperfusion on NCX1 expression were assessed in the rabbit hearts. Compared with either temporally matched, nonischemia-reperfused hearts, or the corresponding nonischemic right ventricular free wall, NCX1 protein expression did not change \( P > \)

Fig. 4. Effect of KB-R7943 (5 μM) on cardiac function (heart rate (HR), left ventricular (LV) systolic and diastolic pressures, \( \pm dP/dt \) and total coronary flow rate (CF) in the isolated rabbit heart. A: diastolic and systolic pressures, HR, and CF; B: \( \pm dP/dt \). KB-R7943 was constantly perfused through the heart beginning at the time indicated. Illustrative data are shown from one heart, were sampled at 1-min intervals, and are representative of data from a total of four hearts.
0.05) in the LV AAR of hearts exposed to 30 min of ischemia and 120 min of reperfusion (Fig. 5).

**DISCUSSION**

Calcium influx via the cardiac NCX has been postulated to play a critical role in myocardial ischemic injury (9, 20, 24, 25, 31). NCX1, the predominant isoform in the heart (17, 21, 22), operates in the reverse mode during ischemia-reperfusion in response to the Na+/H+ exchanger (NHE-1)-dependent elevation in [Na⁺], thus leading to the influx of Ca²⁺ (13, 24). NHE-1 inhibitors, several of which are in clinical trials (zoniporide, cariporide, and eniporide), are cardioprotective and reduce infarct size (8, 12, 14, 23, 26, 30). However, data demonstrating that NCX inhibitors can similarly reduce myocardial infarct size are lacking.

Several studies using nonselective inhibitors, ionic manipulations, or transgenic approaches have implicated NCX in the development of myocardial ischemic injury (6, 9, 20, 25, 31). The identification of the first inhibitor with modest NCX selectivity, KB-R7943 (11), led to studies demonstrating reduced incidence/duration of arrhythmias and enhanced recovery of developed tension in isolated ischemia-reperfused cardiac tissue (16, 19); KB-R7943 also reduced lactate dehydrogenase/creatine kinase release from isolated reperfused hearts (16). While these surrogates may be reflective of cardioprotection in the whole heart, a direct assessment, e.g., infarct size, was not made. Furthermore, the high concentrations of KB-R7943 used (10–20 µM) in these studies also raises some concern as to whether cardioprotection can be obtained with this agent in the whole heart without negative effects on cardiac function (15). More recently, an NCX inhibitor distinct from KB-R7943 and with greater NCX selectivity, SEA0400, was reported to reduce cerebral infarct volume in a middle cerebral artery occlusion model (18); however, its effects on myocardial ischemic injury are unknown.

We observed that KB-R7943 and SEA0400 elicited concentration-dependent reductions in infarct size; moreover, the median cardioprotective concentration of KB-R7943 (0.3 µM) and the SEA0400 EC₅₀ (5.7 nM) were consistent with the reported IC₅₀ for these compounds against NCX (18, 28). Both NCX inhibitors were administered as a constant perfusion beginning before the regional ischemia to mimic a clinical setting in which the compound might be used to reduce injury in a patient known to be at risk for an ischemic event. However, both compounds were equally cardioprotective when administered before reperfusion, indicating that NCX activation upon reperfusion is critical to infarct development and that administration of a NCX inhibitor postischemia would likely be of comparable clinical utility. The cardioprotective efficacy of SEA0400 in particular was similar to that we have previously reported with the NHE-1 inhibitors zoniporide and cariporide, but was greater than that produced by eniporide (maximum reduction in infarct size: 75%, 83%, 85%, and 58% for SEA0400, zoniporide, cariporide, and eniporide, respectively) (14).

Although KB-R7943 reduced infarct size at 0.3 and 1 µM, the cardioprotection achieved was relatively modest (40%), and higher concentrations (5–20 µM) significantly impaired cardiac function (heart rate, LVDP, and ±dP/dt) and CF, leading to eventual failure of the hearts. Early indications of these negative effects on cardiac function were observed at the 1 µM concentration, when LVDP was reduced relative to control during reperfusion, and postischemic recovery of ±dP/dt tended to be depressed. In contrast, whereas SEA0400 also provided concentration-dependent cardioprotection, this compound was more potent than KB-R7943 and reduced infarct size by up to 75%. Furthermore, unlike KB-R7943, SEA0400 was not only free of negative effects on cardiac function at concentrations up to 1 µM, it also significantly improved postischemic ±dP/dt. It is possible that higher concentrations of SEA0400 could negatively impact cardiac function, e.g., as seen with 5 µM KB-R7943, but the limited solubility of SEA0400 precluded our testing of this possibility; nevertheless, given the improved cardiac function observed at 1 µM SEA0400, a negative effect of this compound at only a fivefold higher concentration would appear unlikely.

The present study illustrates that, although NCX inhibitors can reduce infarct size, a clear distinction exists between KB-R7943 and SEA0400, insofar as negative effects on myocardial function would appear to limit the cardioprotective utility of KB-R7943. These compounds are structurally distinct, and, not surprisingly, their pharmacological profiles differ as well (11,
18, 28). In guinea pig ventricular myocytes, KB-R7943 inhibited NCX with an IC50 of 320 nM (28). However, this compound also inhibited voltage-gated Na+ currents, Ca2+ currents, and inward rectifier K+ currents (IC50 of 14, 8, and 7 μM, respectively) (28), resulting in an NCX selectivity of only 20- to 40-fold. Similar studies performed using rat cerebral tissue failed to show any selectivity of KB-R7943 for NCX versus L-type Ca2+ channels, in addition to demonstrating binding affinity for several receptors [α1, β2, muscarinic ACh (mACh)] that modulate cardiac function (it was not reported whether KB-R7943 was an agonist or antagonist at these receptors) (18). Compared with KB-R7943, SEA0400 is a more potent inhibitor of cardiomyocyte NCX activity, inhibiting rat cardiomyocyte NCX with an IC50 of 92 nM (18). Although the selectivity of SEA0400 versus ion channels has not been reported in cardiomyocytes, in rat cerebral tissue, SEA0400 was >360-fold selective versus Na+ channel site 2, the Ca2+ channel dihydropyridine site, and the Ca2+ channel diltiazem site (18); additionally, at an equivalent concentration to KB-R7943 (3 μM), SEA0400 did not bind to α1-, β2-, or mACh receptors. Thus a likely explanation for the differences between KB-R7943 and SEA0400 in our study, i.e., the cardioprotective efficacy of KB-R7943 being limited by impairment of cardiac function, is the minimal selectivity of KB-R7943 for NCX versus other channels ([Na+], [Ca2+], or [K+]) or receptors (α1, β2, or mACh) (18, 28) that could modulate myocardial function. This explanation is supported by our observation that the KB-R7943-dependent impairment of cardiac function only occurred at concentrations (~1 μM) approaching those reported to interact with these channels (7–14 μM) (28) or receptors (3 μM) (18). Furthermore, our data corroborate in vitro studies indicating that concentrations of KB-R7943 ≥0.5 μM inhibit Ca2+ transients and cell shortening in isolated rabbit cardiomyocytes (7, 29) and have negative isotropic effects on isolated dog hearts (15). We also examined whether ischemia-reperfusion affected the levels of NCX1 protein expression in the LV ischemic AAR. On the basis of our pharmacological data, an increase in myocardial NCX1 protein might be expected to contribute to the development of ischemic injury. However, Western blots suggested this was not a factor in our model because NCX1 protein expression did not change after ischemia-reperfusion.

In summary, the NCX inhibitors KB-R7943 and SEA0400 reduced infarct size in an isolated heart model of ischemia-reperfusion injury at concentrations reported to be selective for NCX. Nevertheless, important differences exist between these compounds, as the efficacy of KB-R7943 is modest and limited by a marked depression of cardiac function and changes in CF. At concentrations up to 1 μM, SEA0400 was free of these limitations, produced a greater reduction in infarct size, and improved postischemic dP/dt. Thus NCX inhibitors with a pharmacological profile similar to that of SEA0400 represent an attractive approach for clinically reducing myocardial ischemic injury.

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