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RESEARCH PAPER

Preservation of mitochondrial function may contribute to cardioprotective effects of Na^+/Ca^{2+} exchanger inhibitors in ischaemic/reperfused rat hearts

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Background and purpose: Na^+/Ca^{2+} exchanger (NCX) inhibitors are known to attenuate myocardial reperfusion injury. However, the exact mechanisms for the cardioprotection remain unclear. The present study was undertaken to examine the mechanism underlying the cardioprotection by NCX inhibitors against ischaemia/reperfusion injury.
Experimental approach: Isolated rat hearts were subjected to 35-min ischaemia/60-min reperfusion or 20-min ischaemia/60-min reperfusion. NCX inhibitors (3-30 μM KB-R7943 (KBR) or 0.3-1 μM SEA0400 (SEA)) were given for 5 min prior to ischaemia (pre-ischaemic treatment) or for 10 min after the onset of reperfusion (post-ischaemic treatment).
Key results: With 35-min ischaemia/60-min reperfusion, pre- or post-ischaemic treatment with KBR or SEA neither enhanced post-ischaemic contractile recovery nor attenuated ischaemia- or reperfusion-induced Na^+ accumulation and damage to mitochondrial respiratory function. With the milder model (20-min ischaemia/reperfusion), pre- or post-ischaemic treatment with 10 μM KBR or 1 μM SEA significantly enhanced the post-ischaemic contractile recovery, associated with reductions in reperfusion-induced Ca^{2+} accumulation, damage to mitochondrial function, and decrease in myocardial high-energy phosphates. Furthermore, Na^+ influx to mitochondria *in vitro* was enhanced by increased concentrations of NaCl. KBR (10 μM) and 1 μM SEA partially decreased the Na^+ influx.
Conclusions and implications: The NCX inhibitors exerted cardioprotective effects during relatively mild ischaemia. The mechanism may be attributable to prevention of mitochondrial damage, possibly mediated by attenuation of Na^+ overload in cardiac mitochondria during ischaemia and/or Ca^{2+} overload via the reverse mode of NCX during reperfusion.
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Keywords: ischaemia; mitochondria; Na^+/Ca^{2+} exchanger; reperfusion; oxidative phosphorylation; KB-R7943; SEA0400

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CK, creatine kinase; CP, creatine phosphate; DMSO, dimethyl sulphoxide; HEPs, high-energy phosphates; HR, heart rate; KBR, KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); LVEDP, left ventricular end-diastolic pressure; LVDP, left ventricular developed pressure; NCX, Na^+/Ca^{2+} exchanger; OPR, oxidative phosphorylation rate; RCI, respiratory control index; SEA, SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid; TTX, tetrodotoxin

Introduction

It is generally accepted that ionic disturbances play an important role in the genesis of ischaemia/reperfusion injury (Pierce and Czubryt, 1995). Ischaemia induces Na^+ overload, which is thus far considered to be the driving force of the Ca^{2+} overload that occurs via the Na^+/Ca^{2+} exchanger (NCX) during ischaemia and/or reperfusion (Kleyman and Gragoe, 1988; Tani and Neely, 1989; Meng and Pierce, 1991). The action of the forward mode of the cardiac NCX normally extrudes Ca^{2+} from the cytoplasm to maintain the intracellular Ca^{2+} concentration at its proper level. In contrast, in the case of ischaemia/reperfusion, the action of the reverse mode of the exchanger allows Ca^{2+} influx into cardiac cells as a result of the ischaemia-induced increase in the intracellular Na^+ concentration, thus leading to an intracellular Ca^{2+} overload during reperfusion (Schäfer *et al.*, 2001).

KBR (KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]-isothiourea methanesulphonate) was introduced some years

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ago as a selective NCX inhibitor (Iwamoto *et al*., 1996; Watano *et al*., 1996). This agent is believed to inhibit the action of the reverse mode of the NCX (Iwamoto *et al*., 1996; Watano *et al*., 1996) and thus reduce the accumulation of intracellular Ca^{2+} via NCX in the ischaemic/reperfused heart (Seki *et al*., 2002), resulting in protection of the myocardium against ischaemia/reperfusion-induced contractile failure including myocardial stunning (Yoshitomi *et al*., 2005). In contrast, it has been reported that KBR has a low or poor potency for inhibiting the action of the forward mode of NCX or that of other ion transporters such as K^+ channels, Na^+ channels, Na^+/H^+ exchanger, and L-type Ca^{2+} channels (Iwamoto *et al*., 1996; Watano *et al*., 1996). Recently, another NCX inhibitor, SEA (SEA0400 (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline)), was developed, and it is considered to induce a more selective and potent inhibition of the NCX than KBR (Matsuda *et al*., 2001; Tanaka *et al*., 2002). Although the NCX inhibitors are thought to act by inhibiting NCX activity during ischaemia and reperfusion, further clarification is required.

Recently, we reported that intracellular Na^+ accumulation impaired the ability of mitochondria to generate high-energy phosphates (HEPs) in the ischaemic/reperfused hearts (Iwai *et al*., 2002a), thus leading to contractile dysfunction of the reperfused heart (Yabe *et al*., 2000). These pathophysiological outcomes were attenuated by treatment with Na^+ channel blockers or Na^+/H^+ exchanger inhibitors, preventing mitochondrial damage and improving the function of the ischaemic/reperfused heart (Tani and Neely, 1989; Murphy *et al*., 1991; Van Emous *et al*., 1997; Hartmann and Decking, 1999; Tanonaka *et al*., 2000). These findings suggest that ionic disturbances and/or damage to mitochondrial function during ischaemia/reperfusion may play an important role in the ischaemia/reperfusion injury. In the present study, we examined whether NCX inhibitors might play an important role in the cardioprotection against ischaemia/reperfusion injury by modifying myocardial ion alterations and/or mitochondrial function of the ischaemic/reperfused heart.

Materials and methods

Animals

Male Wistar rats weighing 250–280 g (Japan Laboratory Animals Inc., Tokyo, Japan) were used in the present study. The animals were conditioned to an environment of $23 \pm 1^{\circ}C$, a constant humidity of $55 \pm 5\%$ and a cycle of 12-h light/12-h darkness, and were given free access to food and tap water according to 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). The protocol of this study was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Sciences.

Perfusion of isolated hearts

The perfusion of isolated hearts was performed according to the method described previously (Takeo *et al*., 1995). After anaesthesia of the rats with diethyl ether, their hearts were isolated and perfused at 37°C with a constant flow ($9.0\,ml\,min^{-1}$) of Krebs–Henseleit bicarbonate buffer of the following composition (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.25; $NaHCO_3$, 25 and glucose, 11. The perfusion buffer had been previously equilibrated with a gas mixture of 95% O_2 and 5% CO_2 to pH 7.4. A latex balloon (diameter of 3.7 mm) connected to a pressure transducer (TP-200, Nihon Kohden, Tokyo, Japan) was then inserted into the left ventricular cavity. The initial left ventricular end-diastolic pressure (LVEDP) of the perfused heart was set at 5 mm Hg. The left ventricular developed pressure (LVDP) and heart rate (HR) were monitored via the pressure transducer.

Ischaemia/reperfusion and treatment with KBR or SEA

In the present study, two experimental conditions for ischaemia and reperfusion were set, one in which hearts were subjected to 35 min of ischaemia followed by 60 min of normoxic reperfusion (35-min ischaemia/60-min reperfusion, the first series of experiments) and the other in which 20 min of ischaemia was followed by 60 min of normoxic reperfusion (20-min ischaemia/60-min reperfusion, the second series of experiments). The perfusion of the heart with the Krebs–Henseleit solution was stopped for 20 or 35 min to induce global ischaemia. During the ischaemia, the heart was immersed in this solution pregassed with a mixture of 95% N_2 and 5% CO_2 at 37°C to avoid hypothermia-induced cardioprotection. After 20 or 35 min of ischaemia, the buffer in the organ bath was drained, and the hearts were then reperfused for 60 min under normoxic conditions. The hearts were electrically paced throughout the experiment at the rate of $300\,beats\,min^{-1}$ with an electric stimulator (SEN-3301, Nihon Kohden, Tokyo) except for the first 15 min of reperfusion, to prevent the induction of contractile irregularities that might sometimes occur during this period. KBR and SEA were dissolved in perfusion buffer containing 0.1% dimethyl sulphoxide (DMSO) as a final concentration. DMSO (0.1%) *per se* did not affect any myocardial haemodynamics of the perfused heart. Pre-ischaemic treatment of perfused hearts with KBR or SEA was conducted by infusing the agent into the infusion port just distal to the aortic cannula for the last 5 min before ischaemia at final agent concentrations ranging from 3 to 30 μM or 0.3 to 1 μM, respectively. Post-ischaemic treatment with KBR or SEA was conducted by injection of either agent into the infusion port for the first 10 min of reperfusion, since NCX inhibitors are believed to be substantially effective during reperfusion (Matsumoto *et al*., 2002; Magee *et al*., 2003). We also conducted pre-ischaemic treatment of the ischaemic/reperfused heart with tetrodotoxin (TTX), a Na^+ channel blocker, to compare the effect of TTX with that of NCX inhibitors, because this agent has been demonstrated to protect the perfused heart against ischaemia/reperfusion injury under the present experimental conditions (Iwai *et al*., 2002a).

Creatine kinase activity of the perfusate

The perfusate eluted from the heart during reperfusion following 20- or 35-min ischaemia was collected to estimate

the release of creatine kinase (CK) from reperfused hearts. The CK activity of the perfusate was determined by the method described previously (Iwai *et al*., 2002c).

Determination of myocardial Na^+ and Ca^{2+} contents
Myocardial Na^+ and Ca^{2+} contents were determined by the method described previously (Tanonaka *et al*., 1999). After ischaemia, ischaemia/reperfusion or continuous normoxic perfusion, hearts ($n=5$ each) were perfused with 8 ml of 320 mM sucrose and 20 mM Tris-HCl (pH 7.4), and then the myocardial Na^+ and Ca^{2+} contents were determined by atomic absorption (AA-680, Shimazu, Kyoto, Japan). In a previous study, we extensively characterized this method by using Co^{2+}-EDTA (ethylenediaminetetraacetic acid), which cannot permeate cells. We found that perfusion with 8 ml of sucrose buffer essentially removed ions from the vascular spaces as well as from easily exchangeable extracellular spaces (Tanonaka *et al*., 1999). Thus, the detected ion content represented ions that might have been present in the intracellular milieu and bound to macromolecules or situated within intracellular organelles.

Determination of HEPs
Myocardial HEPs were determined by the method described previously (Takeo *et al*., 1996). After reperfusion or continuous normoxic perfusion, hearts ($n=5$ each) were quickly freeze-clamped with aluminium tongs precooled with liquid nitrogen. Myocardial HEPs were extracted with 0.3 M $HClO_4$ and 0.25 mM EDTA. Each extract was sampled for determination of ATP and creatine phosphate (CP) by high performance liquid chromatography (HPLC) methods.

Preparation of isolated mitochondria
Preparation of cardiac mitochondria was performed by the method described previously (Takeo *et al*., 2004). After ischaemia, reperfusion or continuous normoxic perfusion, hearts were quickly removed from the perfusion apparatus. The heart tissue was homogenized in ice-cold buffer (KEA buffer) containing 180 mM KCl, 10 mM EDTA (pH7.4) and 0.5% fatty acid-free bovine serum albumin (BSA). The homogenate was then centrifuged at 700 *g* for 10 min at 2°C, and the resultant supernatant fluid was centrifuged at 8000 *g* for 10 min at 2°C. The crude mitochondria were again suspended in buffer and centrifuged at 8000 *g* for 10 min at 2°C. The organelles were then resuspended in suspension buffer (20 mM Tris-HCl, pH 6.8, containing 320 mM sucrose and 0.25% BSA) and used for measurement of mitochondrial activity. Protein concentrations were determined by the method of Lowry *et al*. (1951). The isolated mitochondria were used for the measurement of mitochondrial respiratory function and for the determination of Ca^{2+} and Na^+ flux through the mitochondrial membrane *in vitro* as described below.

Measurement of mitochondrial respiratory function
The state 3 and 4 respiration, respiratory control index (RCI), and oxidative phosphorylation rate (OPR) of the mitochondria were determined by the method described earlier (Takeo *et al*., 2004). Isolated mitochondria were incubated in medium consisting of 10 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 10 mM K_2HPO_4 and 10 mM glutamate and stirred at 25°C. The oxygen consumption of the mitochondria was measured in the chamber by using a Clark-type oxygen electrode (Central Kagaku, Tokyo). The quality of the mitochondrial preparation was evaluated by assessing the RCI determined in the presence of 240 nmol ADP.

Na^+ content of mitochondrial fraction in the ischaemic myocardium
We examined mitochondrial Na^+ content of ischaemic hearts with or without the NCX inhibitor treatment to determine whether myocardial Na^+ overload may lead to Na^+ accumulation in mitochondria. After 20- or 35-min ischaemia, hearts were removed from Langendorff apparatus and then myocardial mitochondria were quickly prepared with 320 mM sucrose and 20 mM Tris-HCl (pH 7.4) instead of KEA buffer as described above. The prepared mitochondrial fractions were used for determination of the mitochondrial Na^+ content by using the atomic absorption method.

Ca^{2+} and Na^+ fluxes through mitochondrial membrane in vitro
To elucidate possible mechanisms for the effect of the NCX inhibitors on ionic flux across the cardiac mitochondrial membrane, the following study was performed. Ca^{2+} and Na^+ flux through mitochondrial membranes *in vitro* were determined by the methods of Jung *et al*. (1992) and Cox *et al*. (1993) with some modifications. The membrane-permeable Ca^{2+} indicator Fura-2/AM (acetoxymethyl ester) or Na^+ indicator sodium-binding benzofuran isophthalate-AM (SBFI/AM) was dissolved in DMSO. Since Fura-2/AM and SBFI/AM are hydrophobic probes, 3 μl of 25% (w/v) Pluoronic F-127, a nonionic surfactant, was mixed with 3 μl of either 1 mM Fura-2/AM or SBFI/AM to enhance the loading of the fluorescent cation indicator into the isolated mitochondria. This mixture was then added to 200 μl of incubation buffer of the following composition: 250 mM sucrose, 0.1 mM ethylene glycol tetraacetic acid, 1.5 mM Tris-ATP, 5 mM *N*-Tris(hydroxymethyl)methyl-2-amino sulphonic acid (TES), pH 7.4. The resultant solution was further mixed with 400 μl of incubation medium containing the isolated mitochondria, and then incubated for 30 min at 25°C. After the Fura-2 or SBFI had been loaded into the mitochondria, the suspension was centrifuged at 8000 *g* at 25°C for 5 min to remove any fluorescence probe that had not been incorporated.

Either Fura-2/AM- or SBFI/AM-loaded mitochondria (600 µl) were suspended in a 1-ml cell and then placed in a fluorescence analyzer (CAF110, JASCO, Hachioji, Japan). Ca^{2+}-induced fluorescence signal intensities (excitation at 340 and 380 nm and emission at 500 nm) were monitored in the presence or absence of $CaCl_2$. Na^+-induced fluorescence signal intensities were also measured under the same conditions. Ca^{2+} or Na^+ concentration in the mitochondria was determined as the Fura-2 or SBFI ratio, respectively, which is calculated by the fluorescence intensity obtained

with 340 nm excitation and 500 nm emission relative to that with 380 nm excitation and 500 nm emission (Dosono *et al*., 1992). Ruthenium red was used as an inhibitor of the mitochondrial Ca^{2+} uniporter (Crompton *et al*., 1983; Igbavboa and Pfeiffer, 1988).

Ca^{2+}-induced Ca^{2+} influx to mitochondria

The Fura-2/AM-loaded mitochondria were incubated for 100 s with buffer containing 250 mM sucrose, 5 mM TES, pH 7.4, in the presence of various concentrations of $CaCl_2$ ranging from 1 to 100 μM and then changes in the Fura-2 ratio were determined. The effects of the NCX inhibitors and Ruthenium red on changes in the Fura-2 ratio were also determined in the presence of 30 μM $CaCl_2$.

Na^+-induced Na^+ influx to mitochondria

The SBFI/AM-loaded mitochondria were incubated for 100 s with the same buffer as above at concentrations of NaCl ranging from 6.25 to 50 mM and changes in the SBFI ratio were determined. The effects of various concentrations of the NCX inhibitors on the SBFI ratio were also determined in the presence of 0–50 mM NaCl.

Statistics

The results were expressed as the means $\pm$ s.e.m. Statistical significance was estimated by either analysis of variance (ANOVA) for repeated measures or factorial ANOVA, followed by Fischer's multiple comparison or Dunnett's *t*-test. Differences with a probability of less than 5% were considered to be statistically significant ($P<0.05$).

Drugs

KBR and SEA were kind gifts from Kanebo Pharmaceutical Co. (Osaka, Japan) and Taisho Pharmaceutical Co. (Tokyo, Japan), respectively.

Results

First series of experiments (35-min ischaemia/60-min reperfusion) Changes in LVDP and LVEDP of the ischaemic/reperfused hearts. The time course of changes in the LVDP of ischaemic/reperfused hearts treated with KBR, SEA or TTX is shown in Figure 1. Throughout this paper the term 'untreated' signifies no treatment with inhibitors such as KBR, SEA, TTX and so on but does not refer to ischaemia/reperfusion. Changes in the LVDP were expressed as percentages of the LVDP at the last pre-ischaemia (initial value for LVDP). The initial value for LVDP in the pre-ischaemic, untreated hearts (87.0 $\pm$ 1.5 mm Hg) was not different ($P>0.05$) from that obtained in the hearts treated with 3, 10 and 30 μM KBR or those with 0.3 and 1 μM SEA (89.0 $\pm$ 1.9, 89.0 $\pm$ 1.9, and 87.0 $\pm$ 1.5 mm Hg or 89.4 $\pm$ 4.5 and 93.5 $\pm$ 4.2 mm Hg, $n=5$ each), respectively. After the onset of ischaemia, the LVDP declined to zero within 2.5 min, and it remained at this level during the ischaemia. The LVDP of the untreated heart recovered to 18.0 $\pm$ 1.4% of its initial value by the end of the 60-min reperfusion and the LVDP recovery for the heart treated with 3–30 μM KBR was similar to that for the untreated heart. Similarly, pre-ischaemic treatment with 0.3–1 μM SEA did not enhance the recovery of the LVDP at the end of reperfusion. In contrast, pre-ischaemic treatment with 0.3 μM TTX enhanced the post-ischaemic LVDP recovery to a significant degree.

To determine whether post-ischaemic treatment with the NCX inhibitors would enhance the recovery of cardiac contractile function, we tested KBR and SEA at the concentrations of 3–30 and 0.3–1 μM, respectively. Several reports have shown that these concentrations exerted cardioprotective effects on the perfused heart when administered after ischaemia. However, we found that post-ischaemic treatment with 3–30 μM KBR or 0.3–1 μM SEA did not enhance the post-ischaemic LVDP recovery of the reperfused heart.

Changes in the LVEDP of the ischaemic/reperfused heart are shown in Figure 2. Ischaemia/reperfusion induced a steep rise in the LVEDP during ischaemia followed by a further rise at 5 min after the onset of reperfusion. Subsequent reperfusion maintained the sustained high level of LVEDP throughout the reperfusion. Neither pre-ischaemic nor post-ischaemic treatment with 3–30 μM KBR or 0.3–1 μM SEA significantly modified these changes. In contrast, pre-ischaemic treatment with 0.3 μM TTX significantly attenuated the rise in the LVEDP during the ischaemia and reperfusion.

Spontaneous HR during the first 15 min of reperfusion was monitored. The HRs of untreated hearts at 5-, 10- and 15-min of reperfusion were undetectable, less than 100 beats min^{-1}, and 152 $\pm$ 19 beats min^{-1} ($n=5$), respectively. The HR of the NCX inhibitor-treated heart was similar to that of the untreated, reperfused heart regardless of pre- or post-ischaemic treatment. In contrast, the recovery of HR of the hearts with pre-ischaemic TTX treatment was enhanced in a reperfusion time-dependent manner (108 $\pm$ 23, 205 $\pm$ 32 and 254 $\pm$ 19 beats min^{-1} at 5-, 10- and 15-min of reperfusion, respectively ($n=5$).

Myocardial Na^+ and Ca^{2+} contents at the end of ischaemia and at the end of reperfusion. After measurement of cardiac function, myocardial Na^+ and Ca^{2+} contents were determined at the end of both ischaemia and reperfusion (Figure 3). The baseline values (values for normoxic heart) for myocardial Na^+ and Ca^{2+} contents were 54.4 $\pm$ 0.9 and 1.88 $\pm$ 0.06 μmol g^{-1} dry tissue, respectively. The Na^+ content of untreated hearts increased to approximately 120 μmol g^{-1} dry tissue at the end of the ischaemia. Pre-ischaemic treatment of the heart with 3–30 μM KBR or 0.3–1 μM SEA did not affect this ischaemia-induced increase in Na^+ content. In contrast to the alteration in Na^+ content, there were no significant changes in the Ca^{2+} content at the end of 35-min ischaemia, regardless of treatment, with either agent or untreated.

A further increase in the myocardial Na^+ content was observed during reperfusion. This increase was not attenuated by pre-ischaemic treatment with either 3–30 μM KBR or

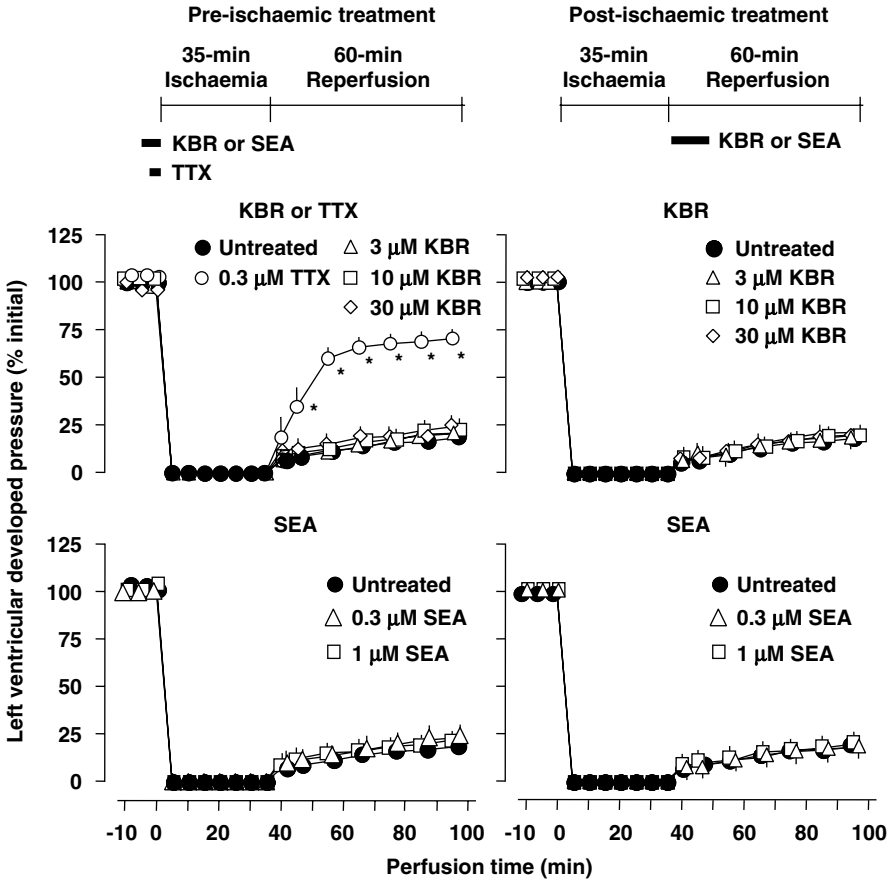

Figure 1 Time course of changes in left ventricular developed pressure (LVDP) of the 35-min ischaemic/60-min reperfused heart untreated or treated with 3, 10 or 30 μM KB-R7943 (upper graphs) or with 0.3 or 1 μM SEA0400 (lower graphs) for the last 5 min of pre-ischaemia (pre-ischaemic treatment in the left graphs). Pre-ischaemic treatment of the ischaemic/reperfused heart with 0.3 μM TTX was also performed for the last 3 min of pre-ischaemia as a reference (the left upper graph). Furthermore, other ischaemic/reperfused hearts were treated with 3–30 μM KBR or 0.3–1 μM SEA for the first 10 min of reperfusion (post-ischaemic treatment in the right graphs). Control values for LVDP of untreated hearts are shown in each graph. Each value represents the mean $\pm$ s.e.m. of five experiments. The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. *Significantly different from the corresponding untreated group ($P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TTX, tetrodotoxin.

0.3–1 μM SEA. The myocardial Ca^{2+} content of the ischaemic/reperfused heart was increased during reperfusion, and this increase was attenuated by treatment with 3–30 μM or 0.3–1 μM SEA in a concentration-dependent manner. It should be noted that the increase in Na^+ during ischaemia as well as during reperfusion and the increase in Ca^{2+} during reperfusion were significantly attenuated in the heart pretreated with 0.3 μM TTX. Post-ischaemic treatment with 3–30 μM KBR or 0.3–1 μM SEA elicited a similar trend in the values for the myocardial Na^+ and Ca^{2+} contents as observed with the pre-ischaemic treatment.

Release of CK from reperfused hearts into perfusate. To determine the activity of CK released from the perfused heart during reperfusion, we collected the perfusate of the heart (Figure 4). During normoxic perfusion, the enzyme activity in the perfusate was approximately 1 nmol NADPH $min^{-1} g^{-1}$ wet tissue. CK activity in the perfusate from the untreated heart markedly increased during reperfusion (more than 150-fold the value for the normoxic group). Treatment with 0.3 μM TTX during pre-ischaemia attenuated the release of the enzyme from reperfused hearts, whereas treatment with neither 10 μM KBR nor 1 μM SEA affected the enzyme release of reperfused hearts regardless of pre- or post-ischaemic treatment.

Mitochondrial respiratory function of ischaemic/reperfused hearts. Table 1 shows the ADP-mediated respiratory function of mitochondria isolated from the agent-untreated-, KBR- or SEA-treated heart at the end of ischaemia or ischaemia/reperfusion. The values for the state 3 respiration and OPR of the untreated heart under the ischaemic condition were significantly decreased to approximately 33 and 30%, respectively, of those for the normoxic heart. The state 3 and OPR of the reperfused heart were further decreased to approximately 20% and almost 0%, respectively, of the values for the normoxic heart ($n=5$). Pre-ischaemic treatment with 10 μM KBR or 1 μM SEA did not recover the state 3 respiration at the end of either ischaemia or reperfusion. A similar trend for changes in the RCI was observed.

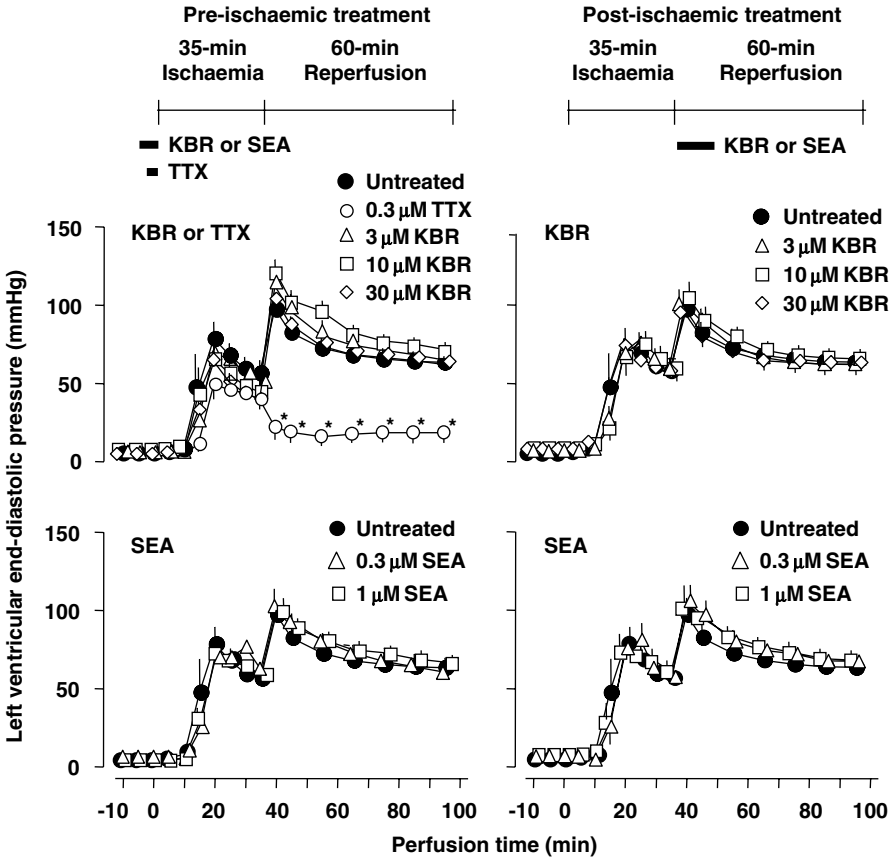

Figure 2 Time course of changes in left ventricular end-diastolic pressure (LVEDP) of the 35-min ischaemic/60-min reperfused heart. The groups are the same as those in Figure 1. Each value represents the mean ± s.e.m. of five experiments. The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. *Significantly different from the corresponding untreated group ($P<0.05$).

Furthermore, in a preliminary study, the values for state 3 respiration of the heart treated pre-ischaemically with 3, 10 and 30 μM KBR or with 0.3 and 1 μM SEA were 28.8 ± 2.6, 30.3 ± 3.0 and 29.1 ± 2.1 nano atoms O min^{-1} per mg protein or 27.3 ± 1.9 and 25.0 ± 1.9 nano atoms O min^{-1} per mg protein, respectively ($n=3$ each), indicating no significant recovery of mitochondrial function by pre-ischaemic treatment with the NCX inhibitors. The mitochondria isolated from the TTX-pretreated heart showed significantly high levels of state 3 respiration, RCI and OPR after 35 min of ischaemia as well as after 60 min of reperfusion as compared with those of the untreated or KBR- or SEA-pretreated hearts.

Post-ischaemic treatment with 10 μM KBR or 1 μM SEA also induced no appreciable recovery of mitochondrial function in the ischaemic/reperfused heart.

HEP contents of ischaemic/reperfused hearts. To determine whether preservation of mitochondrial function might be associated with substantial energy production of the reperfused heart, we measured myocardial HEPs at the end of reperfusion. Figure 5 shows ATP and CP contents of control hearts or those treated with 10 μM KBR or 1 μM SEA during pre-ischaemia. The myocardial ATP and CP contents at 95-min normoxia were 26.16 ± 0.57 and 35.16 ± 1.20 μmol g^{-1} dry tissue ($n=5$), respectively, which were similar to the initial values for ATP and CP (25.14 ± 0.56 and 35.42 ± 1.06 μmol g^{-1} dry tissue, $n=5$, respectively). After 35-min ischaemia/60-min reperfusion, the ATP and CP contents of the untreated heart recovered to approximately 15 and 25%, respectively, of their normoxic values.

When the hearts were pretreated with 10 μM KBR or 1 μM SEA, the ATP and CP contents at the end of reperfusion were similar to those of the untreated heart. However, the heart pretreated with 0.3 μM TTX showed a significant recovery of ATP and CP levels after reperfusion. In a preliminary study, we determined the ATP and CP contents of the ischaemic/reperfused hearts pretreated with 3–30 μM KBR or 0.3–1 μM SEA. The ATP contents of the heart pre-ischaemically treated with 3, 10 and 30 μM KBR or with 0.3 and 1 μM SEA were 4.12 ± 0.45, 3.40 ± 0.55 and 3.59 ± 0.48 μmol g^{-1} dry tissue or 3.89 ± 0.38 and 4.30 ± 0.85 μmol g^{-1} dry tissue, respectively, whereas CP contents were 8.92 ± 0.95, 9.80 ± 1.70 and 9.31 ± 1.04 μmol g^{-1} dry tissue or 8.98 ± 0.38 and 9.76 ± 1.15 μmol g^{-1} dry tissue, respectively ($n=3$ each), indicating no significant recovery of the myocardial HEPs by pre-ischaemic treatment with the NCX inhibitors.

Post-ischaemic treatment with 10 μM KBR or 1 μM SEA did not induce a recovery in the ATP and CP levels of the ischaemic/reperfused heart, similar to the results obtained by the pre-ischaemic treatment.

Second series of experiments (20-min ischaemia/60-min reperfusion)

In the second series of experiments, 20-min of ischaemic period was employed to induce a milder degree of ischaemia/reperfusion injury.

Changes in LVDP and LVEDP of the ischaemic/reperfused hearts. The time course of changes in the LVDP of ischaemic/reperfused hearts treated with 3–30 μM KBR or with 0.3–1 μM SEA is shown in Figure 6. The initial values for the LVDP of the untreated and agent-treated groups ranged

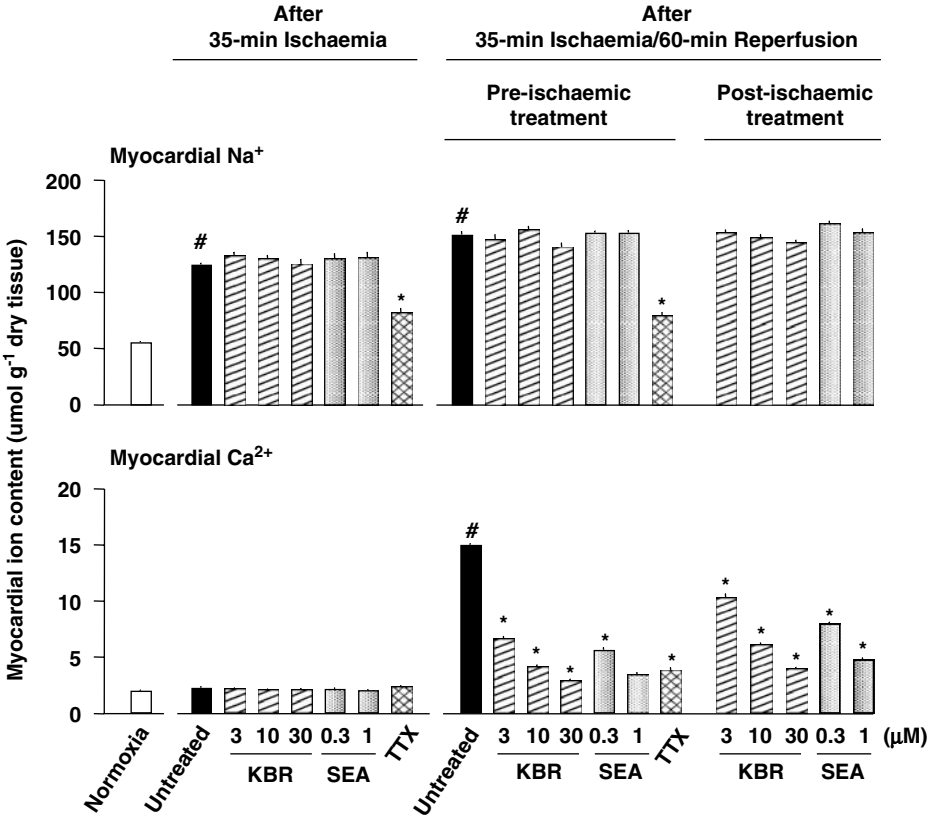

Figure 3 Na^+ (upper graphs) and Ca^{2+} (lower graphs) contents of perfused hearts untreated (untreated) or treated with 3, 10 or 30 μM KB-R7943 (KBR) or with 0.3 or 1 μM SEA0400 (SEA) for the last 5 min of pre-ischaemia (pre-ischaemic treatment), which contents were measured at the end of both ischaemia (after 35-min ischaemia) and reperfusion (after 35-min ischaemia/60-min reperfusion). Other ischaemic/reperfused hearts were treated with 0.3 μM TTX for the last 3 min of pre-ischaemia as a reference. Furthermore, some ischaemic/reperfused hearts were treated with 3–30 μM KBR or 0.3–1 μM SEA for the first 10 min of reperfusion (post-ischaemic treatment). Each value represents the mean$\pm$s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated group ($P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TTX, tetrodotoxin.

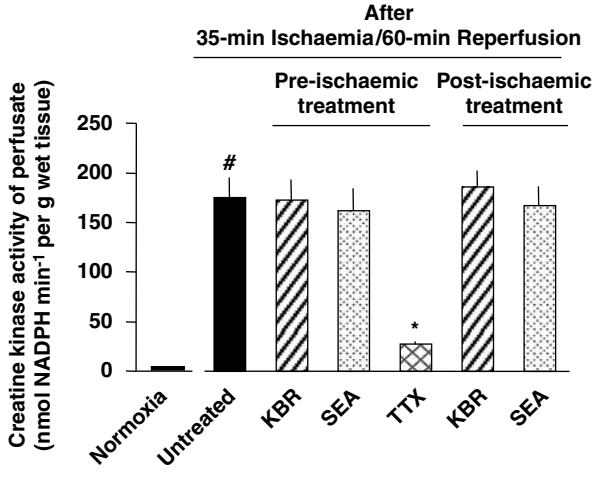

Figure 4 Creatine kinase activity in the perfusate eluted from normoxic (normoxia) or 35-min ischaemic/60-min reperfused hearts untreated (untreated) or treated with 10 μM KB-R7943 (KBR) or 1 μM SEA0400 (SEA) for the last 5 min of pre-ischaemia or with 0.3 μM TTX for the last 3 min of pre-ischaemia (pre-ischaemic treatment; $n=5$ each). Treatment with 0.3 μM TTX was used to provide a reference response. Other ischaemic/reperfused hearts were treated with 10 μM KB-R7943 (KBR) or 1 μM SEA0400 (SEA) for the first 10 min of reperfusion (post-ischaemic treatment). Each value represents the mean$\pm$s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated (untreated) group ($P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TTX, tetrodotoxin.

Table 1 Mitochondrial oxidative phosphorylation of 35-min ischaemic and 35-min ischaemic/60-min reperfused hearts treated with 10 μM KB-R7943, 1 μM SEA0400 or 0.3 μM TTX

	Mitochondrial oxidative phosphorylation			
	State 3 respiration (n atom O min^{-1} mg^{-1} protein)	*State 4 respiration (n atom O min^{-1} mg^{-1} protein)*	*RCI*	*OPR (n mol ATP min^{-1} mg^{-1} protein)*
Normoxia	206.2±4.7	6.5±0.9	33.3±3.6	351.9±18.8
After 35 min ischaemia				
Untreated	68.9±5.2#	12.8±3.6#	6.6±1.2#	104.9±6.3#
10 μm KB-R7943	68.1±4.6#	11.1±0.2	6.4±0.3#	106.2±1.0#
1 μm SEA0400	75.1±6.0#	8.1±0.6	8.9±0.6#	130.2±13.5#
0.3 μm TTX	186.4±3.2#*	8.6±1.4	24.0±4.2#*	296.8±6.1#*
After 35 min ischaemia/60-min reperfusion				
Untreated	37.2±2.6#	Non-detectable	Not calculated	Not calculated
Pre-ischaemic treatment				
10 μm KB-R7943	31.1±3.3#	Non-detectable	Not calculated	Not calculated
1 μm SEA0400	25.1±1.7#	Non-detectable	Not calculated	Not calculated
0.3 μm TTX	152.8±11.0#*	10.3±1.4	15.8±2.4	256.2±18.9
Post-ischaemic treatment				
10 μm KB-R7943	33.6±1.6#	Non-detectable	Not calculated	Not calculated
1 μm SEA0400	35.4±2.9#	Non-detectable	Not calculated	Not calculated

Abbreviations: KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); OPR, oxidative phosphorylation rate; RCI, respiratory control index; SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TTX, tetrodotoxin.
State-3 respiration represents mitochondrial oxygen consumption rate stimulated by the presence of ADP; and state-4 respiration, mitochondrial oxygen consumption rate after cessation of ADP supply. RCI is calculated as the ratio of state-3 respiration/state-4 respiration, and this parameter is used as an index for intactness of the mitochondrial inner membrane. OPR is an index of the ATP-producing ability. Each value represents the mean±s.e.m. of five experiments.
#Significantly different from normoxia group ($P<0.05$). *Significantly different from the corresponding untreated group ($P<0.05$).

from 88.0±2.5 to 92.0±2.0 mm Hg ($n=5$ each). After the onset of ischaemia, the LVDP declined to zero and remained at this level during ischaemia, similar to the results obtained in the first series of experiments. The LVDP of the untreated heart recovered to 60.3±2.0% by the end of the 60-min reperfusion. Pre-ischaemic treatment with 3,10 and 30 μM KBR or with 0.3 and 1 μM SEA significantly recovered the LVDP to 75.6±2.8, 94.7±2.5 and 93.1±2.1 mm Hg or 80.5±3.1 and 94.0±2.1 mm Hg, respectively ($n=5$ each).

Post-ischaemic treatment with the same series of concentrations resulted in a recovery of LVDP to 66.8±3.1, 81.9±1.4 and 80.1±2.2 mm Hg or 72.1±4.0 and 83.2±1.2 mm Hg, respectively ($n=5$ each).

Changes in the LVEDP are shown in Figure 7. Both pre-ischaemic and post-ischaemic treatments with the NCX inhibitors, reduced the ischaemia/reperfusion-induced rise in LVEDP throughout the reperfusion in a concentration-dependent fashion.

The HRs of the NCX inhibitor-untreated hearts at 5, 10 and 15 min of reperfusion were 95±28, 152±33 and 187±21 beats min^{-1} ($n=5$), respectively. The HRs of the heart with pre-ischaemic KBR (10 μM) treatment at 5-, 10- and 15 min of reperfusion was 121±22, 197±21 and 242±20 beats min^{-1} ($n=5$), respectively. The recovery of HR after pre-ischaemic SEA (1 μM) treatment was similar to that of KBR (10 μM)-treated hearts. When the heart was post-ischaemically treated with 10 μM KBR or 1 μM SEA, HR at

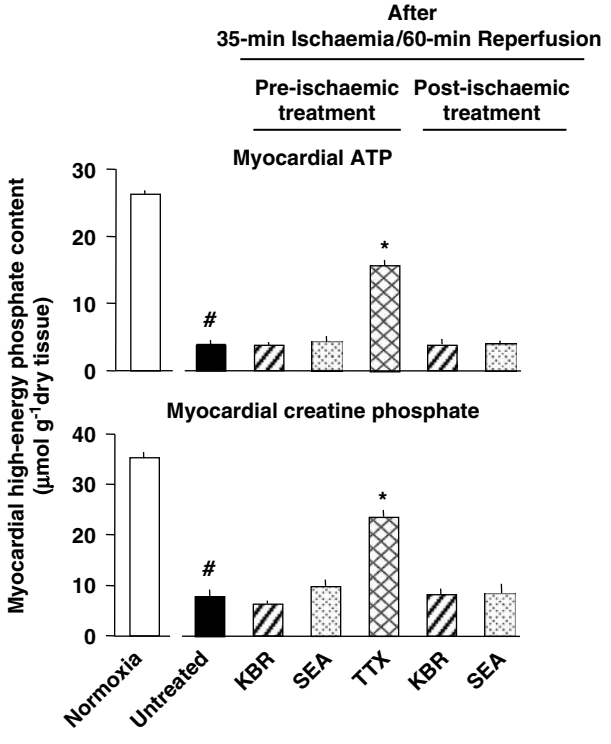

Figure 5 ATP (upper graph) and creatine phosphate (CP; lower graph) contents of 35-min ischaemic/60-min reperfused hearts. The experimental groups are as shown in Figure 4. Each value represents the mean±s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated (untreated) group ($P<0.05$).

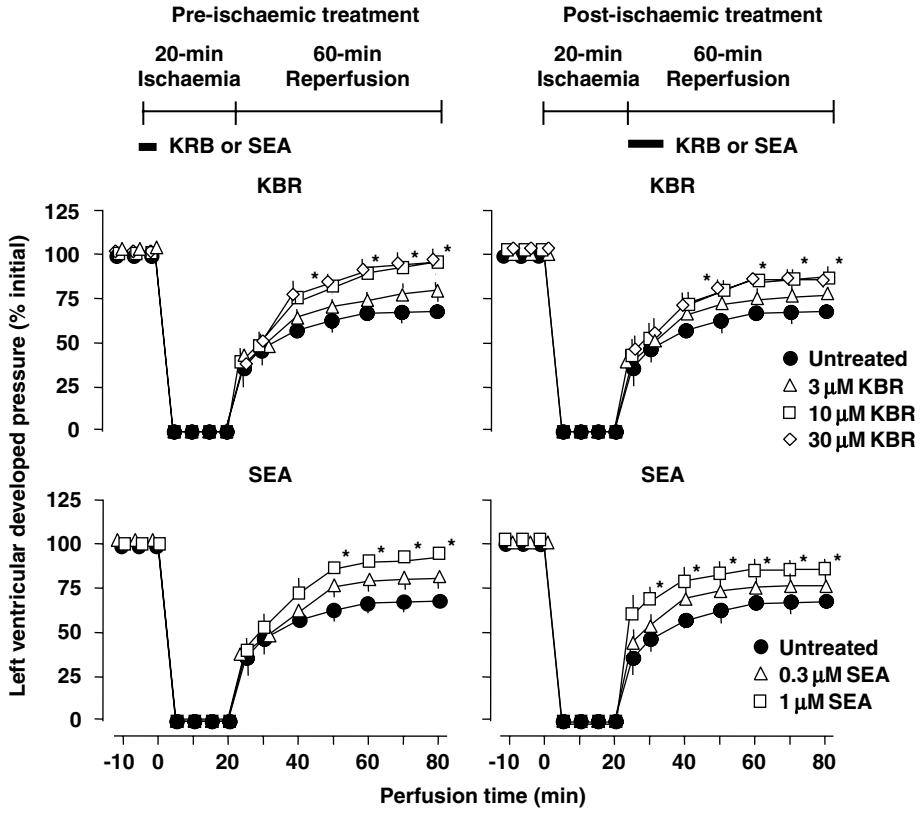

Figure 6 Time course of changes in left ventricular developed pressure (LVDP) of the 20-min ischaemic/60-min reperfused heart untreated or treated with 3, 10 or 30 μM KB-R7943 or with 0.3 or 1 μM SEA0400 for the last 5 min of pre-ischaemia (pre-ischaemic treatment in the left graphs). Other ischaemic/reperfused hearts were treated with 3, 10 or 30 μM KB-R7943 or 0.3 or 1 μM SEA0400 for the first 10 min of reperfusion (post-ischaemic treatment in the right graphs). Each value represents the mean $\pm$ s.e.m. of five experiments. The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. *Significantly different from the untreated ischaemic/reperfused heart ($P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]-phenoxy]-5-ethoxyaniline).

15-min of reperfusion was 206 ± 17 or 211 ± 21 beats min^{-1} ($n = 5$), respectively.

From these results, we focused on the effects of 10 μM KBR and 1 μM SEA in the second series of experiments. We also confirmed the corresponding concentrations among 3–30 μM KBR or 0.3–1 μM SEA to be the best for the recovery of post-ischaemic cardiac function.

Myocardial Na^+ and Ca^{2+} contents at the end of ischaemia and at the end of reperfusion. After measurement of cardiac function, myocardial Na^+ and Ca^{2+} contents were determined at the end of both ischaemia and reperfusion (Figure 8). The Na^+ content of the untreated heart was increased to approximately 80 μmol g^{-1} dry tissue at the end of ischaemia. Pre-ischaemic treatment of the heart with 3–30 μM KBR or 0.3–1 μM SEA did not affect this ischaemia-induced increase in Na^+ content. In contrast to the alteration in Na^+ content, there were no significant changes in Ca^{2+} content at the end of ischaemia in control or treated hearts.

The myocardial Na^+ content of the reperfused heart was similar to that at the end of ischaemia and was not attenuated by treatment with 3–30 μM KBR or 0.3–1 μM SEA. In contrast, the myocardial Ca^{2+} content of the reperfused heart was greatly increased during reperfusion, by about threefold. This reperfusion-induced increase in the myocardial Ca^{2+} content was attenuated by pre-ischaemic treatment with 3–30 μM KBR or 0.3–1 μM SEA.

Post-ischaemic treatment with 3–30 μM KBR or 0.3–1 μM SEA did not attenuate the reperfusion-induced increase in myocardial Na^+, but did reduce the reperfusion-induced increase in myocardial Ca^{2+} in a concentration-dependent manner.

Release of CK from reperfused hearts into perfusate. When perfused hearts were exposed to 20-min ischaemia, CK released from reperfused hearts was increased to approximately 30-fold the value for the corresponding normoxic heart (Figure 9). KBR (10 μM) and 1 μM SEA did not affect the release of the enzyme from reperfused hearts regardless of pre- or post-ischaemic treatment.

Mitochondrial respiratory function of ischaemic/reperfused hearts. Table 2 shows the ADP-mediated respiratory function of mitochondria isolated from the agent-untreated, 10 μM KBR- or 1 μM SEA-treated heart at the end of ischaemia or ischaemia/reperfusion. The state 3 respiration and OPR of

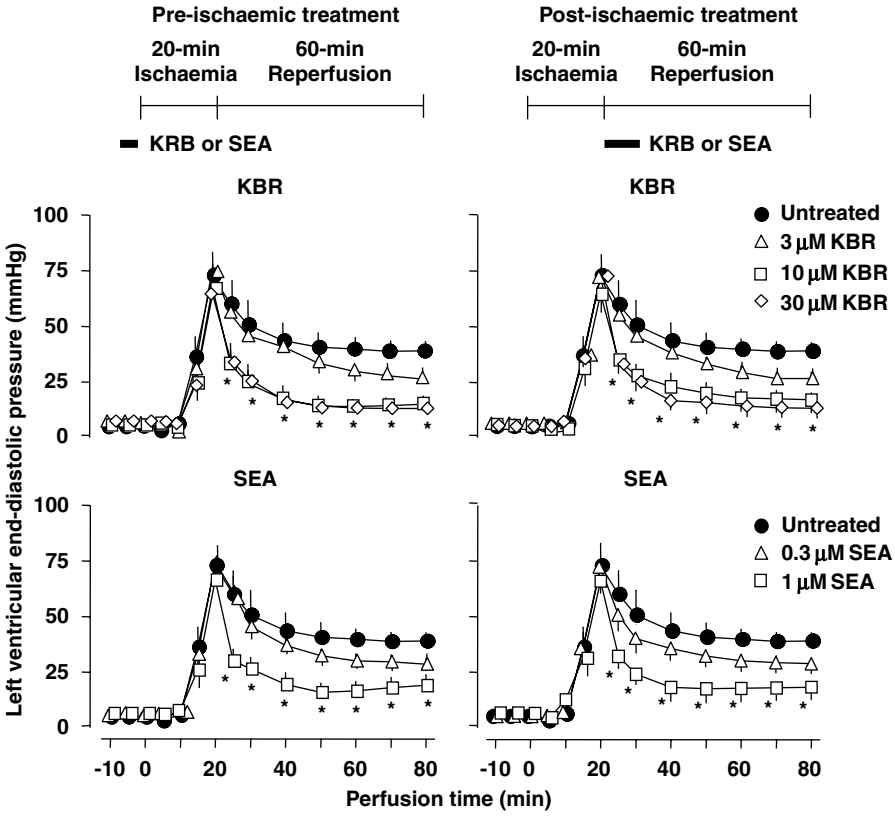

Figure 7 Time course of changes in left ventricular end-diastolic pressure (LVEDP) of the 20-min ischaemic/60-min reperfused heart. The experimental groups are the same as those in Figure 6. Control values for LVEDP of the untreated heart are shown with closed circles in each graph. Each value represents the mean ± s.e.m. of five experiments. The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. *Significantly different from the corresponding untreated group ($P<0.05$).

the untreated heart under ischaemic conditions were significantly decreased to approximately 56 and 70%, respectively, of the values for the normoxic heart ($n=5$ each). The values for both parameters were further decreased to approximately 52 and 55%, respectively, of those for the normoxic heart ($n=5$ each) at the end of reperfusion. Pre-ischaemic treatment with KBR or SEA significantly attenuated the ischaemia- and ischaemia/reperfusion-induced decreases in the values of both state 3 respiration and OPR. A similar trend in changes in the RCI was seen. We also observed in a preliminary study that the state 3 respirations of the hearts at the end of reperfusion treated pre-ischaemically with 3, 10 and 30 μM KBR or with 0.3 and 1 μM SEA were 121.2 ± 4.1, 145.7 ± 5.0 and 148.1 ± 3.1 nano atom O min^{-1} per mg protein or 125.5 ± 2.9 and 142.0 ± 5.5 nano atom O min^{-1} per mg protein, respectively ($n=3$ each) and that the OPRs of the hearts were 208.4 ± 10.6, 259.8 ± 16.0, 285.2 ± 20.1 nmol ATP min^{-1} per mg protein or 218.9 ± 12.2 and 260.1 ± 12.6 nmol ATP min^{-1} per mg protein, respectively ($n=3$ each), indicating concentration-dependent recovery of mitochondrial respiratory function by treatment with the NCX inhibitors.

Post-ischaemic treatment with 10 μM KBR or 1 μM SEA also elicited results similar to those obtained by pre-ischaemic treatment; that is, significant reverses of the state 3 respiration, OPR and RCI of the mitochondria were detected.

HEP contents of ischaemic/reperfused hearts. Figure 10 shows myocardial ATP and CP contents of the reperfused hearts subjected to pre-ischaemic treatment with 10 μM KBR or 1 μM SEA. ATP and CP contents of the continuously normoxic heart at the end of reperfusion were 24.84 ± 0.58 and 34.81 ± 1.07 μmol g^{-1} dry tissue, respectively ($n=5$ each). After the 60-min reperfusion, both ATP and CP contents of untreated hearts were decreased, whereas the ATP and CP content of KBR- or SEA-treated hearts was significantly higher than those of the untreated heart.

We also observed in a preliminary study that myocardial ATP of the 3, 10 and 30 μM KBR-treated or 0.3 and 1 μM SEA-treated, ischaemic/reperfused hearts were 10.98 ± 0.97, 14.05 ± 1.50 and 13.95 ± 1.04 or 10.99 ± 0.87 μmol g^{-1} dry tissue and 13.65 ± 1.80 μmol g^{-1} dry tissue, respectively ($n=3$ each) and that the myocardial CP of the hearts with the same series of concentrations were 24.51 ± 1.96, 28.20 ± 2.10 and 29.66 ± 1.88 μmol g^{-1} dry tissue or 23.99 ± 1.38 and 28.15 ± 2.10 μmol g^{-1} dry tissue, respectively ($n=3$ each). This would indicate a concentration-dependent recovery of myocardial HEPs by pre-ischaemic treatment with the NCX inhibitors.

Post-ischaemic treatment with 10 μM KBR or 1 μM SEA also elicited a significant recovery of myocardial ATP and CP at the end of reperfusion.

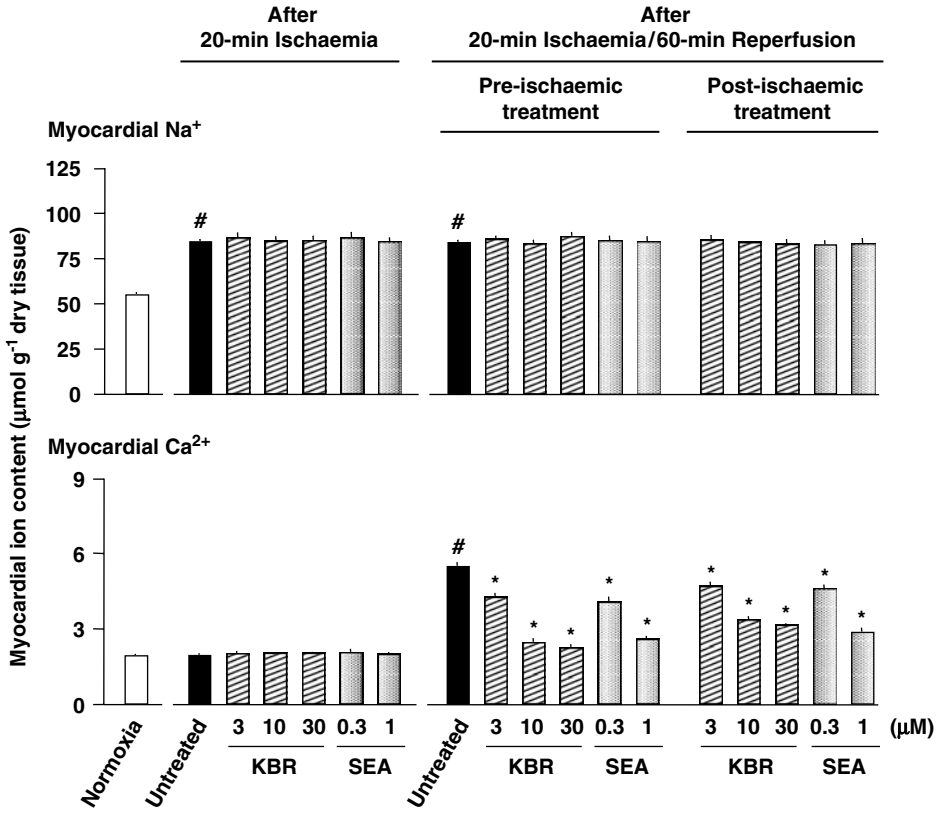

Figure 8 Na^+ (upper graph) and Ca^{2+} (lower graph) contents of perfused hearts untreated (untreated) or treated with 3,10 or 30 μM KB-R7943 (KBR) or with 0.3 or 1 μM SEA0400 (SEA) for the last 5 min of pre-ischaemia (pre-ischaemic treatment), which contents were measured at the end of both ischaemia and reperfusion. Furthermore, other ischaemic/reperfused hearts were treated with 3–30 μM KBR or 0.3–1 μM SEA for the first 10 min of reperfusion (post-ischaemic treatment). Each value represents the mean$\pm$s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated group (untreated; $P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)-methoxy]phenoxy]-5-ethoxyaniline).

Table 2 Mitochondrial oxidative phosphorylation of 20-min ischaemic and 20-min ischaemic/60-min reperfused hearts treated with 10 μM KB-R7943 or 1 μM SEA0400

	Mitochondrial oxidative phosphorylation			
	State 3 respiration (n atom O min^{-1} mg^{-1} protein)	*State 4 respiration (n atom O min^{-1} mg^{-1} protein)*	*RCI*	*OPR (n mol ATP min^{-1} mg^{-1} protein)*
Normoxia	203.7$\pm$11.1	6.6$\pm$1.1	34.2$\pm$5.0	320.6$\pm$18.9
After 20 min ischaemia				
Untreated	113.5$\pm$7.5$^{\#}$	9.9$\pm$1.1$^{\#}$	12.1$\pm$1.9$^{\#}$	223.0$\pm$23.9$^{\#}$
10 μm KB-R7943	149.8$\pm$8.4$^{\#}$*	6.1$\pm$1.0*	27.2$\pm$4.4*	260.2$\pm$17.0$^{\#}$
1 μm SEA0400	152.3$\pm$9.3$^{\#}$*	8.8$\pm$1.3	18.4$\pm$2.2$^{\#}$	285.2$\pm$20.1*
After 20 min ischaemia/60 min reperfusion				
Untreated	106.6$\pm$4.1$^{\#}$	8.7$\pm$1.1	12.9$\pm$1.4$^{\#}$	175.0$\pm$11.6$^{\#}$
Pre-ischaemic treatment				
10 μm KB-R7943	147.5$\pm$4.5$^{\#}$*	5.9$\pm$0.5	25.0$\pm$1.3$^{\#}$*	258.8$\pm$15.3$^{\#}$*
1 μm SEA0400	141.7$\pm$5.1$^{\#}$*	7.6$\pm$1.0	20.4$\pm$3.2$^{\#}$*	255.6$\pm$11.5$^{\#}$*
Post-ischaemic treatment				
10 μm KB-R7943	129.9$\pm$4.8$^{\#}$*	9.8$\pm$1.1$^{\#}$	13.7$\pm$1.0$^{\#}$	236.6$\pm$14.3$^{\#}$*
1 μm SEA0400	130.5$\pm$4.9$^{\#}$*	8.3$\pm$0.5	15.7$\pm$1.3$^{\#}$	223.7$\pm$11.4$^{\#}$*

Abbreviations: KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); OPR, oxidative phosphorylation rate; RCI, respiratory control index; SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline); TTX, tetrodotoxin.
See legend of Table 1 for details.

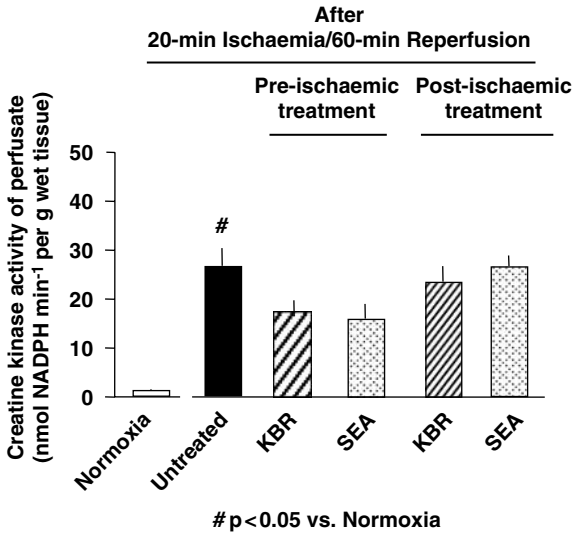

Figure 9 Creatine kinase in the perfusate released from 20-min ischaemic/60-min reperfused hearts untreated or treated with 10 μM KB-R7943 (KBR) or 1 μM SEA0400 (SEA) for the last 5 min of pre-ischaemia (pre-ischaemic treatment), which activity was measured after reperfusion. Other ischaemic/reperfused hearts were treated with 10 μM KB-R7943 (KBR) or 1 μM SEA0400 (SEA) for the first 10 min of reperfusion (post-ischaemic treatment). Each value represents the mean$\pm$s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline).

Effects of NCX inhibitors on mitochondrial Na^+ content in the ischaemic myocardium

Figure 11 shows the effects of pre-ischaemic treatment with 10 μM KBR or 1 μM SEA on changes in mitochondrial Na^+ content at the end of 20- and 35-min ischaemia. The baseline value for the mitochondrial Na^+ content was 10.7 ± 0.6 nmol mg^{-1} protein ($n=4$). The mitochondrial Na^+ content of the agent-untreated heart increased to approximately 20 nmol g^{-1} mg protein at the end of 20-min ischaemia. Pre-ischaemic treatment of the heart with 10 μM KBR or 1 μM SEA attenuated this ischaemia-induced increase in mitochondrial Na^+ content. When the heart was subjected to 35-min ischaemia, the mitochondrial Na^+ content was further increased to approximately 30 nmol mg^{-1} protein. Pre-ischaemic treatment of the heart with the NCX inhibitors partially attenuated the ischaemia-induced increase in mitochondrial Na^+ content.

Experiments using isolated mitochondria in vitro

Na^+-induced Na^+ influx to mitochondria. Figure 12 shows changes in the SBFI signal ratio of isolated mitochondria in the presence of different concentrations of NaCl without any extramitochondrial Ca^{2+} and the effects of KBR (the left graph in Figure 12, $n=4$ each) or SEA (the right graph in Figure 12, $n=4$ each) on the NaCl-induced increase in the SBFI signal ratio. The SBFI signal ratio of the mitochondria was increased by the presence of NaCl in a concentration-dependent manner, which indicates an increase in mitochondrial Na^+ concentration.

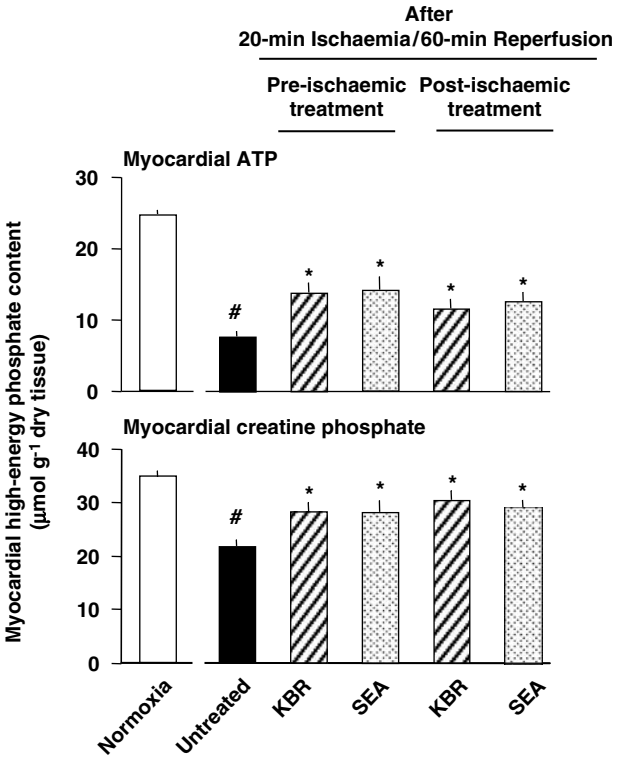

Figure 10 ATP (upper graph) and CP contents (lower graph) of 20-min ischaemic/60-min reperfused hearts. The experimental groups are the same as those in Figure 9. Each value represents the mean$\pm$s.e.m. of five experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated group (untreated; $P<0.05$). CP, creatine phosphate.

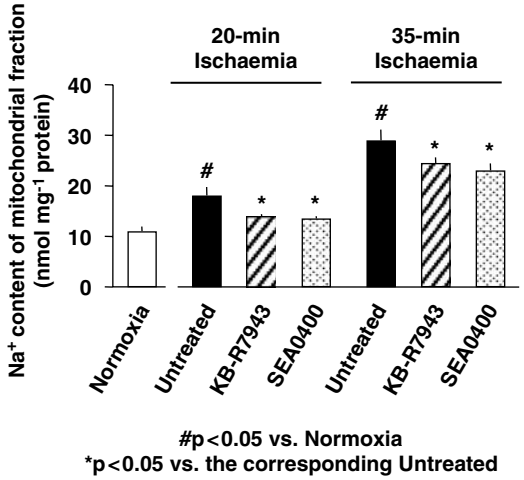

Figure 11 Na^+ content of mitochondria prepared from 20- or 35-min ischaemic hearts untreated (untreated) or treated with 10 μM KB-R7943 (KBR) or with 1 μM SEA0400 (SEA) for the last 5 min of pre-ischaemia. Each value represents the mean$\pm$s.e.m. of four experiments. #Significantly different from the normoxic group (normoxia, $P<0.05$). *Significantly different from the corresponding untreated group ($P<0.05$). KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline).

KBR or SEA at concentrations ranging from 3 to 30 μM or from 0.1 to 1 μM, respectively, did not affect the baseline values for the SBFI signal ratio. The NaCl-induced increase in the mitochondrial SBFI signal ratio was significantly attenuated by the presence of 10 and 30 μM KBR or 0.3 and 1 μM SEA. The maximal attenuation of the SBFI signal ratio in the presence of 25 mM NaCl by these agents amounted to approximately 50% of the total increase, indicating a possible involvement of the NCX inhibitors in the Na^+ influx to mitochondria when the cytosolic Na^+ concentration is increased. The SBFI signal ratio of isolated mitochondria was not affected by the presence of various concentrations of choline chloride ranging from 6.25 to 50 mM under the present experimental conditions (data not shown).

Ca^{2+}-induced Ca^{2+} influx to mitochondria. Figure 13 shows changes in the Fura-2 signal ratio of isolated mitochondria in the presence of various concentrations of $CaCl_2$ without any extramitochondrial Na^+ (the left graph in Figure 13, $n=4$ each) and the effects of KBR, SEA and Ruthenium red on the 30 μM $CaCl_2$-induced increase in the Fura-2 signal ratio (the right graph in Figure 13, $n=4$ each). The Fura-2 signal ratio of the mitochondria was increased at concentrations of 10–100 μM $CaCl_2$. KBR or SEA at any concentrations used did not affect the baseline value for the Fura-2 signal ratio. The right graph in Figure 13 depicts the effects of 10 μM KBR, 1 μM SEA or 10 μM Ruthenium red on 30 μM $CaCl_2$-induced increase in the Fura-2 signal ratio of the mitochondria. The $CaCl_2$-induced increase in the mitochondrial Fura-2 signal ratio was attenuated in the presence of KBR, SEA or Ruthenium red. It should be noted that only 10% of the $CaCl_2$-induced increase in the Fura-2 signal ratio was inhibited by 10 μM KBR or 1 μM SEA, whereas approximately 80% of the $CaCl_2$-induced increase in the Fura-2 signal ratio was abolished by 10 μM Ruthenium red. The results indicate a minor contribution of the NCX inhibitors and a major contribution of Ca^{2+} uniporter inhibitor to Ca^{2+} overload in mitochondria when the cytosolic concentration of Ca^{2+} is increased. The Fura-2 signal ratio of isolated mitochondria was not affected by the presence of various concentrations of choline chloride ranging from 1 to 100 μM under the present experimental conditions (data not shown).

Discussion

In the present study, we employed two experimental conditions to examine the cardioprotective effects of NCX inhibitors. In earlier studies (Liu *et al*., 1993; Tanonaka *et al*., 1999, 2000, 2003; Takeo *et al*., 1995, 2004; Iwai *et al*., 2002a, c), perfusion conditions of 35-min ischaemia and subsequent 60-min reperfusion were usually employed in our laboratory to assess the protective effects of agents on perfused hearts. Several cardiotonic agents, such as TTX, quinidine, lidocaine, flecainide, diltiazem, diazoxide and 2-mercaptopropionyl glycine, were shown to be effective in preventing ischaemia/reperfusion injury under this perfusion condition when the drugs were applied to the heart before ischaemia. For example, with TTX used as a reference in the present study, its cardioprotective effects were evident under the same experimental conditions. Thus, initially we used this condition to determine whether NCX inhibitors might protect the heart against ischaemia/reperfusion injury.

Pre-ischaemic treatment with 3–30 μM KBR or 0.3–3 μM SEA under the 35-min ischaemia/reperfusion conditions failed to enhance the post-ischaemic contractile recovery associated with the release of CK into the perfusate, which is a marker of myocardial necrotic cell death. Also, post-ischaemic treatment neither improved the contractile recovery of the

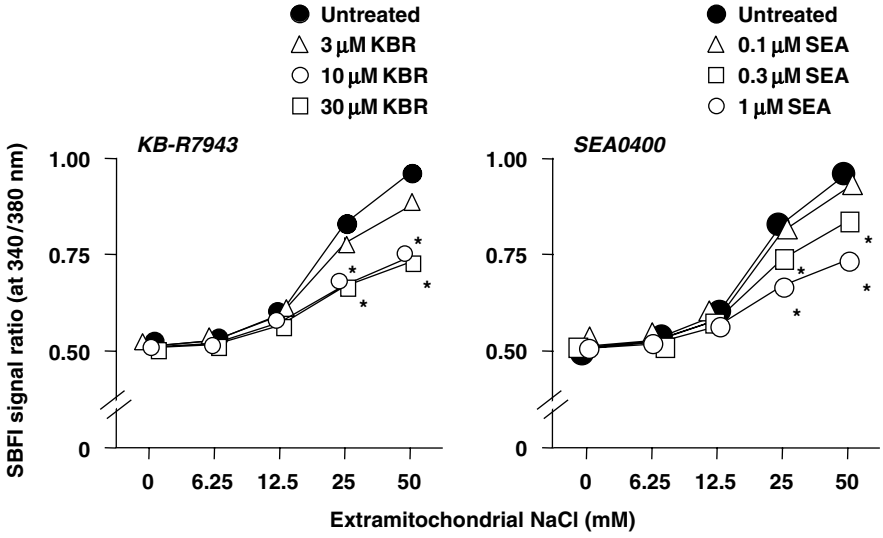

Figure 12 Na^+-induced Na^+ influx to mitochondria *in vitro*. Changes in the SBFI signal ratio (the ratio of 500 nm emission intensities obtained with 340 and 380 nm excitations) of isolated mitochondria at various concentrations of NaCl ranging from 6.25 to 50 mM treated with 3, 10 or 30 μM KBR (left graph) and 0.1, 0.3 or 1 μM SEA (right graph) are shown ($n=4$ each). The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. *Significantly different from untreated group, $P<0.05$. KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)-phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline).

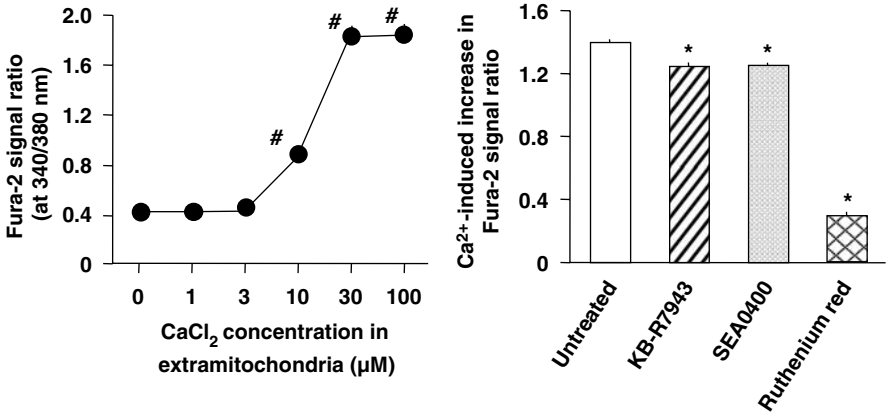

Figure 13 Ca^{2+}-induced Ca^{2+} influx to mitochondria *in vitro*. Changes in the Fura-2 signal ratio (the ratio of 500 nm emission intensities obtained with 340 and 380 nm excitations) of the isolated mitochondria in the presence of different concentrations of $CaCl_2$ (left graph) and the effects of 10 μM KB-R7514 (KBR), 1 μM SEA0400 (SEA) and 10 μM Ruthenium red on the 30 μM $CaCl_2$-induced increase in the Fura-2 signal ratio (right graph) are shown ($n=4$ each). The 30 μM $CaCl_2$-induced increase in Fura-2 signal ratio shown in the right graph was determined by subtracting the baseline value for the Fura-2 fluorescence intensity from the 30 μM $CaCl_2$-induced fluorescence intensity. The s.e.m. for the symbols without a bar are within 3% and masked by the symbols. #Significantly different from the basal value (the value for the group without $CaCl_2$). *Significantly different from untreated group (untreated) $P<0.05$. KB-R7943, (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate); SEA0400, (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline).

reperfused heart nor attenuated the release of CK from the reperfused heart, but did attenuate myocardial Ca^{2+} accumulation during reperfusion, similar to the results of the pre-ischaemic treatment. Several reports have shown beneficial effects of treatment with KBR or SEA on ischaemic/reperfused rat (Nakamura *et al*., 1998; Takahashi *et al*., 2003) and rabbit (Magee *et al*., 2003) hearts, on low-flow/reperfusion rat hearts (Seki *et al*., 2002), and on *in vivo* ischaemia/reperfusion rat and dog hearts (Yoshiyama *et al*., 2004; Yoshitomi *et al*., 2005). Thus, there appears to be a discrepancy between our results and those of others as to the effects of NCX inhibitors on the ischaemic/reperfused heart. Post-ischaemic recovery of the LVDP of the untreated heart under the 35-min ischaemia/60-min reperfusion conditions was approximately 18% as compared with the pre-ischaemic LVDP. Conversely, the post-ischaemic LVDP recoveries of the untreated and ischaemic/reperfused hearts by other investigators were more than 50% of the pre-ischaemic value (Takahashi *et al*., 2003). Since more than 50% recovery of the LVDP was observed in hearts subjected to shorter than 20-min ischaemia followed by 60-min reperfusion (Iwai *et al*., 2002b), we next examined the effects of the NCX inhibitors on the ischaemic/reperfused heart under the 20-min ischaemia/60-min reperfusion conditions. As a result, we found a slight but significant enhancement of post-ischaemic contractile recovery of the reperfused heart after pre-ischaemic treatment with the NCX inhibitors. Thus, the discrepancy may be attributed to the experimental conditions employed. Accordingly, it is likely that NCX inhibitors may exert cardioprotective effects under relatively mild ischaemia/reperfusion conditions. It is considered that there are significant species differences in NCX activity of cardiomyocytes: the NCX activity is higher in human and rabbit ventricles than in rat and mouse hearts (Sham *et al*., 1995; Bers, 2002). We cannot rule out this possibility for the difference in the cardioprotective effect of the NCX inhibitors.

What is the mechanism responsible for cardioprotection by NCX inhibitors in the ischaemic/reperfused heart? We focused on ionic disturbances in the ischaemic/reperfused heart, because severe ischaemia/reperfusion injury has been shown earlier to be associated with massive accumulation of Na^+ and Ca^{2+} in the perfused heart (Iwai *et al*., 2002a,b). In both series of experiments, we observed marked increases in the myocardial Na^+ content during ischaemia and ischaemia/reperfusion and in the myocardial Ca^{2+} content during reperfusion. Apparently, in the present study, the myocardial Na^+ and Ca^{2+} contents that were measured did not represent their free ion state in the cytosol and/or mitochondria (Tanonaka *et al*., 1999), as described in Methods section. Rather, the alterations in ion contents were the sum of several ionic movements during ischaemia/reperfusion via the NCX, Na^+/H^+ exchanger, Na^+/K^+ pump, Na^+ channels, Ca^{2+} channels, and Ca^{2+} uniporter in cellular or subcellular membranes. Thus, stoichiometric analysis of the cardiac ion concentrations cannot be assessed from the results in the present study. Despite this uncertainty, we detected no significant change in the myocardial Ca^{2+} content at the end of 20- or 35-min ischaemia as compared with the initial value, whereas a significant increase in the myocardial Ca^{2+} content was observed at the end of 60-min reperfusion following 20- or 35-min ischaemia. Thus, a massive Ca^{2+} accumulation occurred during reperfusion. This idea is essentially consistent with the results of Hagihara *et al*. (2005).

In the present study, we observed that the NCX inhibitors concentration-dependently reduced Ca^{2+} accumulation during reperfusion in both 35-min ischaemic/60-min reperfused and 20-min ischaemic/60-min reperfused hearts. Thus, it is conceivable that attenuation of Ca^{2+} overload during reperfusion through inhibition of the reverse mode of NCX in cardiac cell membranes is a mechanism for cardioprotective effects of the NCX inhibitors. Nevertheless, our study failed to show the preventive effect of the NCX inhibitors

against the contractile dysfunction of the 35-min ischaemic/60-min reperfused heart. Furthermore, in the isolated mitochondrial study, we observed a large inhibition of Ca^{2+} influx to the mitochondria by a Ca^{2+} uniporter inhibitor Ruthenium red. In contrast, although the NCX inhibitors also attenuated Ca^{2+} influx in the isolated mitochondria, their effects were markedly smaller than that of Ruthenium red. Thus, the Ca^{2+} uniporter, rather than the mitochondrial NCX, may play an important role in Ca^{2+} influx into mitochondria when the cytosolic concentration of Ca^{2+} is increased. These results obtained from the studies on Ca^{2+} movement in isolated mitochondria and in perfused hearts suggest that another mechanism different from the inhibition of NCX activity might be mainly involved in the cardioprotective effect of the NCX inhibitors under the present experimental conditions.

In previous studies (Iwai *et al*., 2002b; Takeo *et al*., 2004), we hypothesised that cytosolic Na^+ overload directly caused mitochondrial Na^+ overload, which resulted in damage to mitochondrial function during ischaemia as well as during reperfusion, and eventually led to contractile dysfunction in the ischaemic/reperfused heart. These events are consistent with the results in the first series of experiments and comparable with our observation that pre- or post-ischaemic treatment of the ischaemic/reperfused heart with the NCX inhibitors ameliorated the mitochondrial oxidative phosphorylation activity and thereby enhanced the recovery of myocardial HEPs in the reperfused heart in the second series of experiments.

We observed that mitochondrial oxidative phosphorylation activity of the heart pre-ischaemically treated with NCX inhibitors was significantly higher at the end of ischaemia than that of the untreated heart. Despite such difference in the mitochondrial activity, Na^+ accumulation during ischaemia and reperfusion was similar between untreated and NCX inhibitor-treated hearts in the second series of experiments, providing further insight into the distribution of Na^+ overload within a cell.

In the experiment using isolated mitochondria *in vitro*, we observed an increase in Na^+-induced Na^+ influx to mitochondria and its attenuation by the NCX inhibitors. This finding suggests that myocardial Na^+ overload leads to mitochondrial Na^+ overload and that NCX inhibitors are capable of attenuating the Na^+ shift to mitochondria under ischaemic or reperfusion conditions. In fact, we have shown that mitochondrial Na^+ content is increased in an ischaemic time-dependent manner. In the heart subjected to 20-min ischaemia, the NCX inhibitors may appreciably reduce Na^+ influx to mitochondria and then preserve the mitochondrial energy-producing ability. However, since the NCX inhibitors incompletely attenuated the mitochondrial NCX activity as shown in Figure 12, Na^+ in mitochondria was markedly accumulated under sustained ischaemic conditions, that is, 35-min ischaemia. It is conceivable that this attenuation in the Na^+ influx by the NCX inhibitors may lead to less damage to mitochondrial phosphorylation, when Na^+ accumulation is relatively small as seen under 20-min ischaemic conditions. Thus, it is suggested that a prevention of Na^+ influx to the mitochondria, followed by preservation of the mitochondrial ability to produce HEPs plays an important role in the amelioration of cardiac functional and metabolic recovery in the reperfused heart treated pre-ischaemically or post-ischaemically with the NCX inhibitors KBR and SEA.

From the above results, we conclude that Na^+ overload during ischaemia induces mitochondrial damage depending on the duration of the ischaemia. When the mitochondrial damage is severe, NCX inhibitors do not provide effective cardioprotection during ischaemia or during reperfusion. Rather, Na^+ channel blockers can potently exert cardioprotective effects. In contrast, when the mitochondrial damage is mild, NCX inhibitors are effective in affording cardioprotection, possibly by attenuating Na^+ overload to mitochondria during ischaemia and/or Ca^{2+} overload via the reverse mode of NCX during reperfusion in the perfused rat heart.

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Conflict of interest

The authors state no conflict of interest.

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