KB-R7943 inhibits the mitochondrial Ca\(^{2+}\) uniporter but not Na\(^+\)-Ca\(^{2+}\) exchanger in cardiomyocyte-derived H9c2 cells

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Summary

The effect of KB-R7943, an inhibitor of the plasmalemmal Na\(^+\)-Ca\(^{2+}\) exchanger, on mitochondrial Ca\(^{2+}\) transporters was examined with membrane-permeabilized cardiomyocyte-derived H9c2 cells expressing the fluorescent Ca\(^{2+}\) indicator, yellow cameleon 3.1, in the mitochondria. KB-R7943, as well as ruthenium red, inhibited the rise in mitochondrial Ca\(^{2+}\) on increasing the extramitochondrial Ca\(^{2+}\) concentration from 0 nM to 300 nM. CGP37157, but not KB-R7943, inhibited the decline in mitochondrial Ca\(^{2+}\) on return to Ca\(^{2+}\) free extramitochondrial solution. These results indicated that KB-R7943 has inhibitory effects on the mitochondrial Ca\(^{2+}\) uniporter, but not on the mitochondrial Na\(^+\)-Ca\(^{2+}\) exchanger.

Keywords: mitochondria, Ca\(^{2+}\) uniporter, Na\(^+\)-Ca\(^{2+}\) exchanger, Ca\(^{2+}\) imaging, KB-R7943, cardiomyocytes
Introduction

The $\text{Na}^+\text{-Ca}^{2+}$ exchanger (NCX) is involved in the regulation of $\text{Ca}^{2+}$ concentration in the myocardium. The plasmalemmal NCX basically functions in the forward ($\text{Ca}^{2+}$ efflux) mode, but under pathological conditions such as ischemia-reperfusion, it functions in the reverse ($\text{Ca}^{2+}$ influx) mode and causes cellular damage through cellular $\text{Ca}^{2+}$ overload.\(^1\) The two major NCX inhibitors with benzyloxyphenyl structure, KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulfonate) and SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-ethoxyaniline), have been used as potent pharmacological tools in NCX research.\(^2\)

Earlier studies have shown that NCX inhibitors have protective effects against ischemia-reperfusion injury in several organs including the heart. In models of myocardial ischemia-reperfusion, KB-R7943 and SEA0400 reduced cytoplasmic and mitochondrial $\text{Ca}^{2+}$ overload and enhanced the recovery of contractile force and ATP content;\(^3\)\(^-\)\(^6\) this could be explained by reduction of $\text{Ca}^{2+}$ entry into the cytoplasm through plasmalemmal NCX. These results suggested that inhibition of plasmalemmal NCX protects the mitochondria through attenuation of cytoplasmic $\text{Ca}^{2+}$ overload during myocardial ischemia.

Another possible explanation for the mitochondrial protective effects of NCX inhibitors was their direct action on mitochondrial $\text{Ca}^{2+}$ transport, either reduction of $\text{Ca}^{2+}$ entry or enhancement of $\text{Ca}^{2+}$ extrusion. To examine such possibility, we established cardiomyocyte-derived H9c2 cells expressing the $\text{Ca}^{2+}$ sensitive fluorprobe yellow cameleon 3.1.
in the mitochondria, which enabled observation of mitochondrial Ca$^{2+}$ entry and Ca$^{2+}$ extrusion.

Using this system, we previously reported that SEA0400 affects neither mitochondrial Ca$^{2+}$ entry nor Ca$^{2+}$ extrusion.$^{7,8}$ Concerning the effect of KB-R7943 on mitochondrial Ca$^{2+}$ transporters, conflicting results have been reported depending on the cell type.$^{9,10}$ In the present study, we intended to clarify the effect of KB-R7943 on mitochondrial Ca$^{2+}$ transport in cardiomyocytes using the H9c2 cell system described above.

Methods

The experimental system was basically the same as that described in our previous reports.$^{7,8}$ Rat embryonic heart derived H9c2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. To obtain mitochondrial-targeted yellow cameleon DNA, its full-length cDNA$^{11}$ was inserted into an expression vector which has a mitochondria targeting signal sequence, pEYFP-mito (Clontech, Palo Alto, CA, USA). This vector was introduced into H9c2 cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and stable transformants were obtained by clone culture in the presence of G418, a neomycin analogue. For the measurement of mitochondrial function, the plasmalemmal membrane was permeabilized by perfusion of digitonin (20 µg/ml) in a Ca$^{2+}$-free solution that contained (in mM) 50 KCl, 80 potassium aspartate, 4 sodium pyruvate, 20 HEPES, 3 MgCl$_2$, 3 Na$_2$ATP, 5.8 glucose, and 3 EGTA (pH 7.3 with KOH). The free Ca$^{2+}$ concentration in the extramitochondrial solution...
was changed to 300 nM according to the experimental protocol. The cells were observed with an epifluorescence microscope (IX70, Olympus). The cells were excited at 440 nm and the emissions at 480±30 nm and 535±25 nm were detected by a cooled CCD camera (HISCA, Hamamatsu Photonics) at a time resolution of 5 s, and ratioed after correction of background fluorescence (Aquacosmos software, Hamamatsu Photonics). The inhibitors used were KB-R7943 (Nihon Organon, Osaka, Japan), ruthenium red (Sigma, St. Louis, MO, USA), and CGP37157 (Tocris, Bristol, UK). All data were expressed as means ± SEM. Data was analyzed by the one-way analysis of variance followed by Dunnett's multiple tests. If the P value was less than 0.05, the difference was considered statistically significant.

Results

In permeabilized H9c2 cells, raising the extramitochondrial Ca\textsuperscript{2+} concentration from 0 nM to 300 nM resulted in an increase in fluorescence ratio, which indicated an increase in mitochondrial Ca\textsuperscript{2+} concentration. On return to Ca\textsuperscript{2+} free extramitochondrial solution, the fluorescence ratio declined toward its initial value indicating a decrease in mitochondrial Ca\textsuperscript{2+} concentration. The rise and fall of fluorescence ratio was reproducible which enabled evaluation of pharmacological effects.

The increase in fluorescence ratio caused by raising the extramitochondrial Ca\textsuperscript{2+} concentration from 0 nM to 300 nM was used as an index of mitochondrial Ca\textsuperscript{2+} uniporter activity (Fig. 1). Ruthenium red (1 μM), an inhibitor of the mitochondrial Ca\textsuperscript{2+} uniporter, had no
effect on the fluorescence ratio under Ca\(^{2+}\) free extramitochondrial solution. The rise in fluorescence ratio by 300 nM extramitochondrial Ca\(^{2+}\) was markedly inhibited by ruthenium red, which confirmed the involvement of the Ca\(^{2+}\) uniporter. KB-R7943 (10 \(\mu\)M) had no effect on the fluorescence ratio under Ca\(^{2+}\) free extramitochondrial solution, but significantly inhibited the rise in fluorescence ratio by 300 nM extramitochondrial Ca\(^{2+}\).

The decline in fluorescence ratio on return to Ca\(^{2+}\) free extramitochondrial solution was used as an index of the mitochondrial NCX activity (Fig. 2). CGP37157 (10 \(\mu\)M), an inhibitor of the mitochondrial NCX, had no effect on the fluorescence ratio under 300 nM extramitochondrial Ca\(^{2+}\). The decline in fluorescence ratio on return to Ca\(^{2+}\) free extramitochondrial solution was markedly inhibited by CGP37157, which confirmed the involvement of mitochondrial NCX. KB-R7943 (10 \(\mu\)M) affected neither the fluorescence ratio under 300 nM extramitochondrial Ca\(^{2+}\) nor the decline in fluorescence ratio on return to Ca\(^{2+}\) free extramitochondrial solution.

**Discussion**

The present study was undertaken to clarify whether KB-R7943 has direct effects on mitochondrial Ca\(^{2+}\) transport in cardiomyocytes. We used membrane-permeabilized cardiomyocyte-derived H9c2 cells expressing the fluorescent Ca\(^{2+}\) indicator, yellow cameleon 3.1 in the mitochondria, which was proven to be useful for the evaluation of the mitochondrial effects of SEA0400.\(^{7,8}\)
The mitochondrial Ca\textsuperscript{2+} uniporter is considered to be the main system for Ca\textsuperscript{2+} influx into the mitochondria. This transporter is inhibited by ruthenium red or its derivative Ru360.\textsuperscript{12) } Our previous study with H9c2 cells indicated that the elevation of mitochondrial Ca\textsuperscript{2+} concentration caused by an increase in extramitochondrial Ca\textsuperscript{2+} concentration was inhibited by ruthenium red and carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) which eliminates the driving force of the Ca\textsuperscript{2+} uniporter, but enhanced by CGP-37157, a mitochondrial NCX inhibitor.\textsuperscript{8) } In our preliminary experiments, cyclosporine A, which inhibits the opening of the mitochondrial permeability transition pore (PTP), had no effect on the increase in mitochondrial Ca\textsuperscript{2+}. These results indicated that Ca\textsuperscript{2+} influx mainly occurs through the Ca\textsuperscript{2+} uniporter.

Whether KB-R7943 affects mitochondrial Ca\textsuperscript{2+} uptake is controversial; KB-R7943 inhibited the Ca\textsuperscript{2+} uniporter in Hela cells,\textsuperscript{9) } while it did not in AD293 cells;\textsuperscript{10) } this suggests that the pharmacological property of the Ca\textsuperscript{2+} uniporter may differ among cell types. Our present results indicated that KB-R7943 inhibits the Ca\textsuperscript{2+} uniporter in H9c2 cells. We also confirmed that KB-R7943 did not depolarize the mitochondrial membrane in cardiomyocytes (data not shown). Thus, the presently observed inhibitory effect of KB-R7943 on mitochondrial Ca\textsuperscript{2+} uptake was unlikely to be an indirect effect through mitochondrial depolarization. The inhibition of mitochondrial Ca\textsuperscript{2+} uptake by KB-R7943 probably is an advantage for the treatment of ischemia-reperfusion injury. In fact, ruthenium red was reported to reduce mitochondrial Ca\textsuperscript{2+}
overload and prevent cell death in cardiomyocytes.\textsuperscript{13} As KB-R7943 is membrane permeable, it is accessible to the mitochondrial Ca\textsuperscript{2+} uniporter when applied extracellularly in intact cells or tissue preparations. Thus, blockade of the mitochondrial Ca\textsuperscript{2+} uniporter probably contributes to the mitochondria protective action of KB-R7943.

The mitochondrial NCX is considered to be the main system for extrusion of mitochondrial Ca\textsuperscript{2+} into the cytoplasm. This transporter is encoded by the NCLX gene which has 62\% homology with the plasmalemmal NCX in their transmembrane repeats $\alpha 1$ and $\alpha 2$.\textsuperscript{14} Mitochondrial NCX can be discriminated from plasmalemmal NCX by their pharmacological properties; while SEA0400 selectively inhibits plasmalemmal NCX, CGP37157, a benzothiazepine compound, selectively inhibits mitochondrial NCX.\textsuperscript{7,15} The present results showed that KB-R7943 does not inhibit mitochondrial NCX. Whether this is an advantage for the treatment of ischemia-reperfusion injury awaits further investigation. It was reported in the rat heart that inhibition of the mitochondrial NCX by clonazepam was deleterious under ischemia-reperfusion.\textsuperscript{16}

The two major NCX inhibitors, KB-R-7943 and SEA0400, block the forward and reverse modes of the plasmalemmal NCX, but not mitochondrial NCX. These inhibitors, especially KB-R7943, has additional blocking effect on plasmalemmal voltage dependent ion channels\textsuperscript{17} and intracellular transporters.\textsuperscript{10} In addition, the present study indicated that KB-R7943 inhibits the mitochondrial Ca\textsuperscript{2+} uniporter cardiomyocyte-derived H9c2 cells. Such
profiles should be taken into consideration when applying these NCX inhibitors to assay systems and pathological models.

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Conflict of Interest The authors declare no conflict of interest.
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Figure 1: Effect of KB-R7943 on mitochondrial Ca$^{2+}$ entry in H9c2 cells. The permeabilized H9c2 cells expressing mitochondria-targeted yellow cameleon 3.1 were permeabilized and fluorescence ratio was measured as an index of intramitochondrial Ca$^{2+}$ concentration. The cells were kept in a Ca$^{2+}$ free extramitochondrial solution and exposed to extramitochondrial solution containing 300 nM Ca$^{2+}$ for 5 minutes twice with a 10 minutes interval. Before the second elevation of Ca$^{2+}$, the cells were untreated with agents (control; A), treated with 1 μM ruthenium red (B) or treated with 10 μM KB-R7943 (C). Summarized results of fluorescence ratio before and after the second application of 300 nM Ca$^{2+}$ were presented (D). Data points with vertical bars indicate the mean ± S.E.M. from 4-6 cells. Asterisks indicate significant differences (P < 0.05) from the corresponding control values.
Figure 2: Effect of KB-R7943 on mitochondrial Ca\(^{2+}\) extrusion in H9c2 cells. The permeabilized H9c2 cells were kept in a Ca\(^{2+}\) free extramitochondrial solution and exposed to extramitochondrial solution containing 300 nM Ca\(^{2+}\) for 5 minutes. Before return to 0 nM Ca\(^{2+}\) extramitochondrial solution, the cells were untreated with agents (control; A), treated with 10 μM CGP37157 (B) or treated with 10 μM KB-R7943 (C). Summarized results of fluorescence ratio before and after the application of Ca\(^{2+}\) free extramitochondrial solution were presented (D). Data points with vertical bars indicate the mean ± S.E.M. from 5-6 cells. Asterisks indicate significant differences (P < 0.05) from the corresponding control values.