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KB-R9032, newly developed Na^+/H^+ exchange inhibitor, attenuates reperfusion-induced arrhythmias in isolated perfused rat heart

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Abstract
Purpose. This study was conducted to elucidate the effects of KB-R9032, a newly developed Na^+-H^+ exchange inhibitor, on reperfusion-induced ventricular arrhythmia in the isolated perfused rat heart.
Methods. Male Wistar rat hearts ($n = 48$; 12 for each group) were perfused with modified Krebs-Ringer's solution equilibrated with 5% carbon dioxide in oxygen by means of the Langendorff technique. An occluder was placed around the left anterior descending coronary artery (LAD). Heart rate, coronary flow, and ECG were monitored. Drug-free perfusate was used for 10 min before switching to a perfusate containing various concentrations of KB-R9032. The added concentrations of KB-R9032 varied in the range of 0 (control) to 1×10^{-5} mol·l^{-1}. Each heart was subjected to regional ischemia (occlusion of LAD for 11 min) and to 3 min of reperfusion (release of the ligation).
Results. In the control group, reperfusion-induced ventricular fibrillation (VF) occurred in 91.7%, and the duration was 158.2 ± 14.4 s (mean ± SEM); however, 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·l^{-1} KB-R9032 reduced the incidence of VF to 75.0%, 42.9%, and 6.7%, respectively ($P < 0.05$ at 1×10^{-5} mol·l^{-1} of KB-R9032) and reduced the duration of VF to 64.8 ± 22.1, 16.8 ± 10.1, and 1.2 ± 1.2 s, respectively ($P < 0.05$ at 1×10^{-6} and 1×10^{-5} mol·l^{-1} of KB-R9032).
Conclusion. It was shown in this study that the Na^+/H^+ exchange inhibitor KB-R9032 suppresses reperfusion arrhythmias in the ischemia-reperfusion model of isolated rat heart.

Key words Reperfusion · Arrhythmia · Ischemia-reperfusion cardiac injury · Na^+-H^+ exchange inhibitor · KB-R9032

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Introduction

Reperfusion of the ischemic heart, achieved by therapies such as coronary artery bypass grafting, thrombolysis, and percutaneous coronary angioplasty has become a standard technique in patients with myocardial ischemia. It is very important to avoid reperfusion injury to improve the outcome of these reperfusion therapies. Reperfusion-induced arrhythmias are a specific phenomenon that can be specifically and reproducibly induced immediately after reperfusion, and this has been well established [1] as an experimental model of myocardial injury associated with ischemia-reperfusion. To date, a number of different theories for the pathogenesis of these ischemia-reperfusion arrhythmias have been proposed, and many factors involved in the mechanism have been studied [2]. Since the early experiments by Jennings et al. [1], it has been noted that Ca^{2+} is associated with reperfusion injuries. It is now widely accepted that cytoplasmic Ca^{2+} increases significantly on reperfusion, and that the calcium overload is closely associated with the pathogenesis of reperfusion injuries.

It has recently been reported [3] that the intracellular Na^+ concentration increases prior to the Ca^{2+} overload, and the roles of the intra- and extracellular kinetics of H^+ and Na^+ through the Na^+/H^+ and Na^+/Ca^{2+} exchangers have been emphasized as the cause of the Ca^{2+} overload. In this context, it is now considered that the imbalance in the distribution of these ions results in Ca^{2+} overload at various stages of the ischemia-reperfusion pathophysiological mechanism. Therefore, there has been an increasing recognition of the importance of the roles of the Na^+/H^+ exchangers in the myocardial ischemia-reperfusion sequence. In this regard, inhibitors of Na^+/H^+ exchangers have attracted attention because they offer a possible way to control Ca^{2+} overload by modulating the kinetics of these ions.

Although many experimental data support the usefulness of Na^+/H^+ exchange inhibitors, there have been no such clinically successful drugs. KB-R9032, a new drug that has been developed as a potent inhibitor of Na^+/H^+ exchange, has hydrophilic properties. Its highly water-soluble property is expected to be helpful in clinical practice. If this drug is capable of sufficiently inhibiting the intracellular Ca^{2+} overload, it would possibly minimize and suppress the occurrence of reperfusion arrhythmias, and it may be possible to test this in an experimental model where these arrhythmias may be reliably reproduced.

Thus, in this study, we investigated the effects of KB-R9032 on reperfusion arrhythmias in the ischemia-reperfusion model of isolated rat heart.

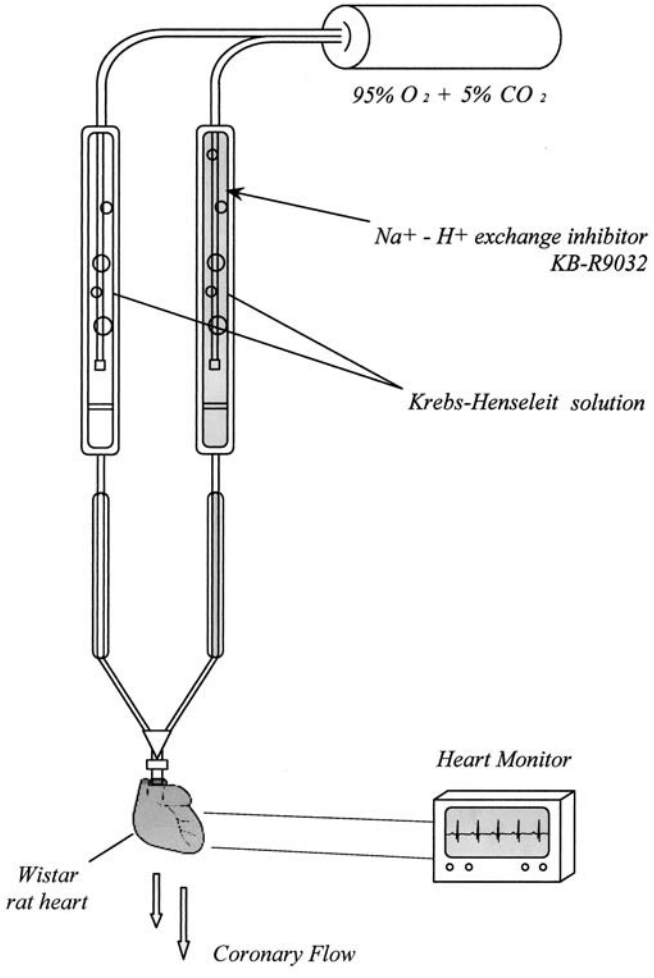


Fig 1. Schematic diagram of the experiment

Materials and methods

Animals

Male Wistar rats, weighing 270–300 g (9 weeks old) were purchased from Clea Japan (Osaka, Japan) and were maintained for 1 week with 12-h lighting in an air-conditioned room at a temperature of 22 ± 2°C, and 50 ± 10% humidity. Food pellets (CE-2; Clea Japan) and water were available ad libitum. Forty-eight male Wistar rats at the age of 10 weeks were divided into four groups (12 animals per experimental group). All animal experiments were performed in accordance with the animal protection and control laws and the standards of care and management of experimental animals.

Isolation and perfusion of rat hearts

Rats were anesthetized with ether for 7 min and subsequently injected with 200 units of sodium heparin through the femoral vein. After an additional 1 min of anesthesia with ether, the heart was dissected, cooled in a perfusate at 4°C, and perfused by means of the Langendorff technique [4]. The perfusate used was Krebs-Henseleit (KH) buffer ($mmol \cdot l^{-1}$: NaCl, 118.5; $NaHCO_3$, 25; KCl, 3.2; $KHPO_4$, 1.19; $MgSO_4$, 1.18; $CaCl_2$ $6H_20$, 2.5; glucose, 11) saturated with a mixture of 95% O_2 + 5% CO_2. The perfusion pressure was 100 cmH_2O, and the perfusate was filtered through a cellulose-acetate membrane (0.3 μm pore size) prior to use. The electrical activity of the heart was monitored with an electrocardiograph (HR-3; San-Ei Electronics, Tokyo, Japan) with the electrodes attached to the cardiac apex and aortic root, and recorded with a pen recorder (Recticorder; Nihon Kohden, Tokyo, Japan) at 25 $mm \cdot s^{-1}$ to monitor the heart rate and occurrence of arrhythmias. One vessel was filled with KH buffer only, and the other one was filled with KH solution containing KB-R9032 at different concentrations, as described below (Fig. 1).

Induction of ischemia and reperfusion

A 5-0 polyvinyl suture (5-0 Nespyren; Azwell, Osaka, Japan) was positioned loosely around the left anterior descending coronary artery (LAD) at approximately 3 mm distal to the origin of the vessel, with both ends threaded through a plastic "occluder" tube. All experiments were started after aerobic perfusion with oxygenated KH solution for 10 min in order to stabilize the heart rate and the coronary flow. Coronary ligation was produced by traction of the occluder against the LAD, and reperfusion was achieved by undoing the ligation with the occluder.

KB-R9032 and its concentration

KB-R9032 (FW, 400.46) was kindly provided by Nippon Organon (Tokyo, Japan). Solutions of KB-R9023 were made by dissolving the reagent in KH solution at concentrations of 1×10^{-7} mol·l^{-1}, 1×10^{-6} mol·l^{-1}, and 1×10^{-5} mol·l^{-1}. Four groups of animals were used for experiments, consisting of the above three KB-R9032 groups and a control group where the hearts were perfused with KH solution without drug.

Experimental protocol

For equilibration, the isolated hearts were perfused for an initial 10 min with KH solution that did not contain KB-R9032. Thereafter, the perfusate was switched to the KH solutions containing the different concentrations of KB-R9032, noted above, and perfusion was continued for an additional 10 min. Simultaneous controlled experiments were conducted without adding KB-R9032 (the control group). After the perfusion fluid was changed once, it was not changed again throughout the experiment. Following aerobic perfusion, regional ischemia was produced by ligation of the LAD for 11 min. The heart was then reperfused by releasing the tourniquet around the LAD. Reperfusion was continued for 3 min. All experiments were performed in the four groups of animals, which included the control (C group; perfused with KH solution only) and three KB-R9032 groups (perfused with KH solutions containing 10^{-7}, 10^{-6}, and 10^{-5} mol·l^{-1}, respectively).

Indices measured

Coronary flow (CF) was measured as the output from the pulmonary artery, using a 10-ml measuring cylinder. Heart rate (HR) was measured from the ECG tracing. Both indices were measured during the last 1 min of perfusion without KB-R9032 (time point 1), during perfusion with the drug (time point 2), after 11 minutes of coronary occlusion (time point 3), and after 3 min of reperfusion (time point 4). Measurements were not made for the hearts that did not show sinus rhythm at time point 4.

Analysis of arrhythmias

When the perfused hearts exhibited ventricular fibrillation (VF) before the reperfusion, the experiment was stopped at the onset of VF and the data from the specimen were excluded from the analysis of reperfusion arrhythmias.

During the 3-min reperfusion, ECG was continuously recorded at 25 mm·s^{-1}, and the induced ventricular arrhythmias were classified as ventricular tachycardia (VT), ventricular fibrillation (VF), and ventricular premature contraction, according to the guidelines of the Lambeth Conventions [5]. The incidence of VT was defined as the ratio (percentage) of experiments where VT occurred during the observation period in relation to the whole number of experiments in that group. The duration of VT was defined as the cumulative time in seconds (s) of all VT events observed during the time interval; the incidence of VF and the duration of VF were defined as for VF.

Statistical analysis

All data values for HR, CF, duration of VT, and duration of VF are presented as means ± SEM, and statistical comparisons among the four groups and within a group (HR, CF) were performed with the Kruskal-Wallis test. When this test indicated a significant difference, the data were analyzed further with Scheffe's test. Fisher's direct probability test was used for statistical analysis of the incidence of VT and VF. Statistical significance was accepted at the 5% level ($P < 0.05$).

Results

None of the perfused hearts exhibited VF prior to reperfusion; therefore, no experiments were excluded from the study ($n = 12$ per group; 48 in total). The changes in CF are shown in Table 1. In all experimental groups, CF was decreased significantly by coronary occlusion and was restored to the pre-occlusion level by reperfusion, and there were no significant differences between the KB-R9032-treated groups and the control,

Table 1. Changes in coronary flow (ml·min $^{-1}$)

	Time point 1	Time point 2	Time point 3	Time point 4
Control	10.7 ± 0.4	10.1 ± 0.5	7.2 ± 0.5*:**:***	12.0 ± 0.5
1×10^{-7}	11.2 ± 0.4	9.7 ± 0.3	5.9 ± 0.5*:**:***	11.1 ± 0.6
1×10^{-6}	11.8 ± 0.5	10.2 ± 0.4	7.0 ± 0.4*:**:***	12.3 ± 0.7
1×10^{-5}	10.8 ± 0.7	10.4 ± 0.7	7.7 ± 0.7*:**:***	11.7 ± 0.8

* $P < 0.05$ vs time point 1; ** $P < 0.05$ vs time point 2; *** $P < 0.05$ vs time point 4
Values are expressed as means ± SEM
Time point 1, before administration of KB-R9032; time point 2, after administration of KB-R9032; time point 3, after 11 min of coronary occlusion; time point 4, after 3 min of reperfusion

Table 2. Changes in heart rate (beats·min $^{-1}$)

	Time point 1	Time point 2	Time point 3	Time point 4
Control	263 ± 4	240 ± 7	237 ± 5	241 ± 6
1×10^{-7}	274 ± 7	239 ± 10	213 ± 15	256 ± 11
1×10^{-6}	266 ± 7	226 ± 7	218 ± 7	225 ± 9
1×10^{-5}	290 ± 7	255 ± 9	223 ± 9	222 ± 8

Values are expressed as means ± SEM
Time point 1, before administration of KB-R9032; time point 2, after administration of KB-R9032; time point 3, after 11 min of coronary occlusion; time point 4, after 3 min of reperfusion. The values of time point 4 were calculated using only rat hearts in normal sinus rhythm at the end of the 3-min reperfusion period

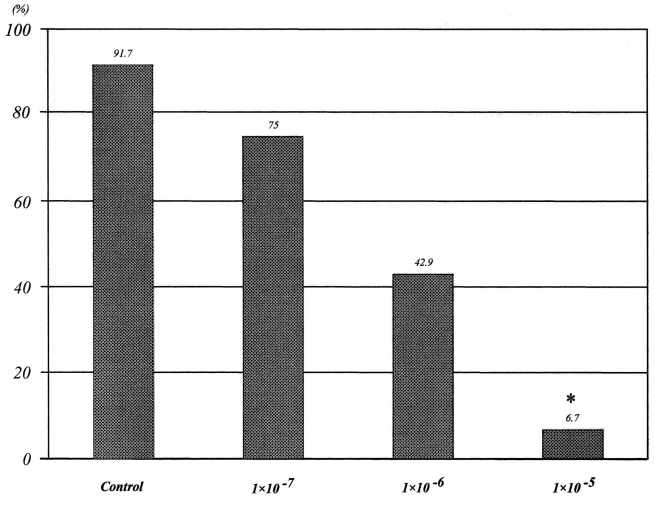


Fig 2. Effects of KB-R9032 on the incidence of ventricular fibrillation. * $P < 0.05$ vs control

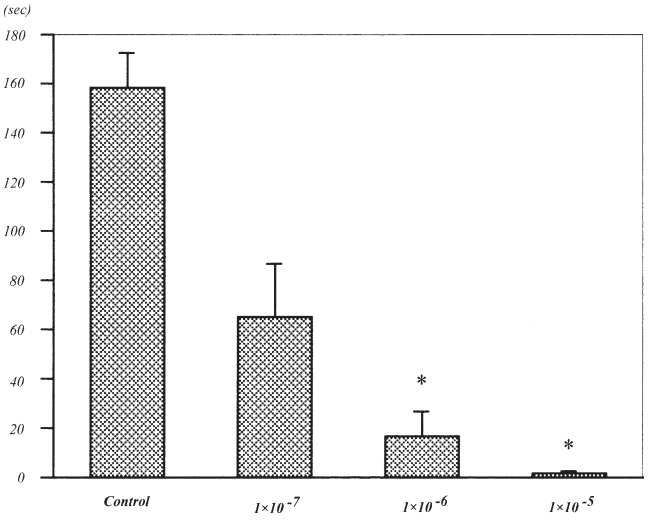


Fig 3. Effects of KB-R9032 on the duration of ventricular fibrillation. * $P < 0.05$ vs control

or between the various KB-R9032-treated groups at any time point (Table 1). Also, no significant difference in HR was noted between the groups at any time point (Table 2).

In the control group (C group), VT occurred in 100% of the hearts; by contrast, the incidences of VT in the KB-R9032-perfused groups were 66.7%, 53.6%, and 60% for 1×10^{-7}, 1×10^{-6} and 1×10^{-5} mol·l^{-1}, respectively. However, the differences were not statistically significant compared with the C group.

The duration of VT in the C group was an average of 13.2 s, while the KB-R9032-perfused groups exhibited average times of 14.0, 3.9, and 7.6 s for 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·l^{-1} KB-R9032, respectively. The duration was significantly shorter in the 1×10^{-6} mol·l^{-1} group compared with the C group.

The incidence of VF was 91.7% , 75.0%, 42.9%, and 6.7% for the C group and 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·l^{-1} for the KB-R9032-perfused groups, respectively. The difference between the C group and the 1×10^{-5} mol·l^{-1} group was statistically significant (Fig. 2). The average duration of VF in the C group was 158.2 s, and the KB-R9032-perfused groups had average durations of 64.8, 16.8, and 1.2 s for 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} mol·l^{-1} KB-R9032, respectively. The duration was significantly shorter in the 1×10^{-6} and 1×10^{-5} mol·l^{-1} KB-R9032-perfused groups compared with the C group (Fig. 3).

Discussion

Our results demonstrated that the newly developed Na^+/H^+ exchange inhibitor, KB-R9032, reduced the incidence of reperfusion-induced ventricular arrhythmias in perfused rat hearts.

Recent progress in coronary reperfusion therapy has resulted in an inevitable increase in ischemia-reperfusion conditions, thereby attracting more attention from researchers to examine its pathophysiology. Clinically, ventricular arrhythmias associated with ischemia-reperfusion are not seen as frequently as

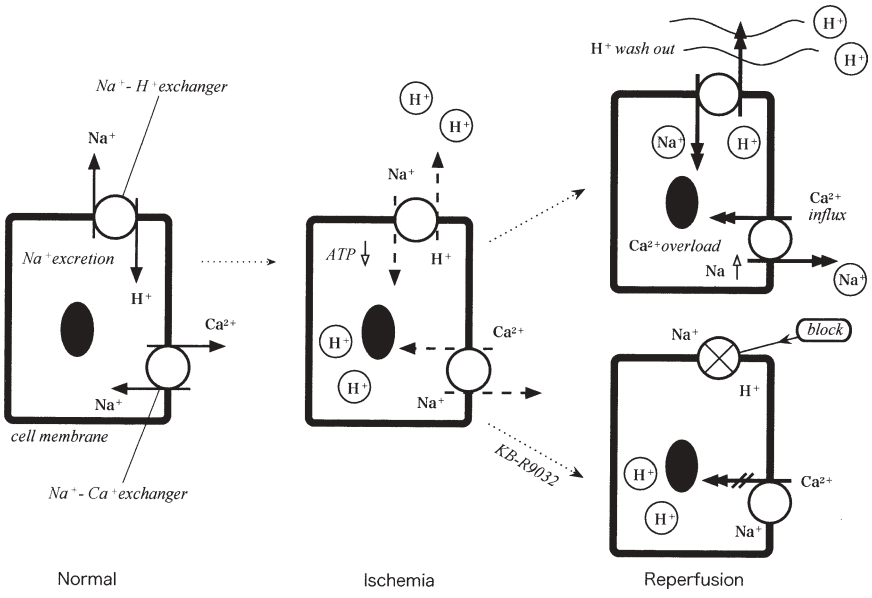


Fig 4. Schematic illustration of Na +-H+ exchange and Na +-Ca2+ exchange in cardiac myocytes

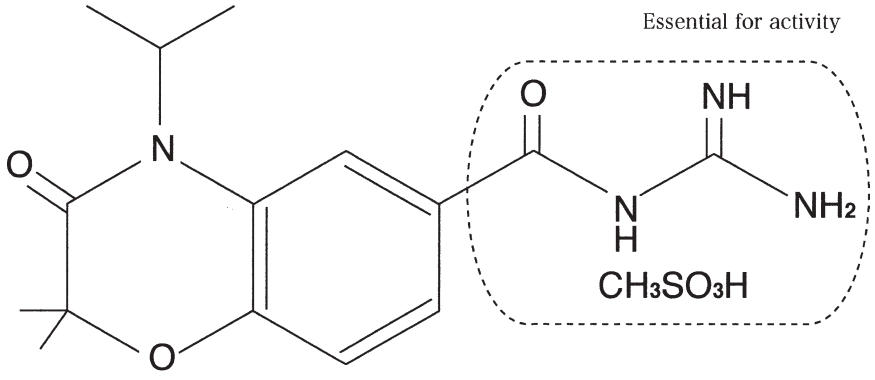
in animal experiments; however, these experimental arrhythmias are a specific phenomenon that can be specifically and reproducibly induced immediately after reperfusion, and this has been well established [6] as an experimental model of myocardial injury associated with ischemia-reperfusion. In particular, the reperfusion-induced arrhythmias in isolated perfused hearts have been extensively studied [7–9].

In a series of prior experiments as groundwork, we investigated the reproducibility of this experimental model, along with the frequency and the optimal period for observation of reperfusion-induced arrhythmias. According to the preliminary studies, we determined to set the duration of ischemia in this study as 11 min [10]; this time interval optimally stabilizes the heart rate and the coronary flow and induces arrhythmia at the highest frequency in the perfused heart.

As with the pathogenesis of reperfusion-induced arrhythmias, it can be said that most pathophysiological processes that trigger the reperfusion-induced arrhythmias are connected to intracellular Ca^{2+} overload [2,3,11]. Recently, it has been elucidated that Na^+/H^+ and Na^+/Ca^{2+} exchangers play a key role in the Ca^{2+} overload observed during the period of reperfusion. Under the ischemic condition, both Na^+/H^+ and Na^+/Ca^{2+} exchangers remain inactive. However, when the reperfusion is introduced, the Na^+/H^+ exchange starts functioning in the reverse direction to normal, allowing H^+ to pass to the outside and Na^+ to enter inside the cell. In conjunction with a rapid increase in the intracellular Na^+ concentration, the Na^+/Ca^2 exchanger also starts working in reverse, resulting in a rapid influx of Ca^{2+} into the cell. This rapid rise of Ca^{2+} is thought to result in the reperfusion-induced arrhythmias. It has been reported that there is an increase in intracellular Na^+ prior to the Ca^{2+} overload associated with the ischemia-reperfusion sequences [3]. If the Na^+/H^+ exchange is inhibited, the rapid influx of Ca^{2+} is blocked during the reperfusion period. So, reperfusion-induced arrhythmias can be avoided. The scheme of events discussed above may explain the mechanism of the inhibitory effect of the Na^+/H^+ exchange inhibitor on reperfusion-induced arrhythmias (Fig. 4).

Inhibition of the Na^+/Ca^{2+} exchanger may be more efficient in the prevention of arrhythmias, as it directly suppresses an increase in intracellular Ca^{2+} concentration. In fact, it has been reported that Na^+/Ca^{2+} exchange inhibitors suppressed reperfusion-induced arrhythmias [12,13], but, because these agents directly block the Ca^{2+} influx, this may have a risk of inhibiting cardiac function itself. As Na^+/H^+ exchange inhibitors do not have any direct effects on cardiac function, they are thought to be more advantageous than inhibitors of the Na^+/Ca^{2+} exchanger in the prevention of reperfusion-induced arrhythmias. However, the exchange of Na^+ and H^+ could possibly exacerbate the intracellular acidosis associated with ischemia. The effect of the acidosis on cardiac function awaits further investigations.

Although the effects of various Na^+/H^+ exchange inhibitors, such as amiloride, or amiloride derivatives such as 5-(N-ethyl-N-isopropyl) amiloride (EIPA), and 4-isopropyl-3-methyl-sulfonylbenzoyl guanidine methanesulfonate (HOE), on reperfusion injuries have already been reported [14,15] in different models of cardiac reperfusion in experimental animals, these agents may still have some limitations for wide clinical application because of their hydrophobic properties. So



KB-R9032

N-(4-isopropyl-2,2-dimethyl-3-oxo-3,4-dihydro-2H-benzo[1,4]oxazine-6-carbonyl)guanidine · MsOH

Fig 5. Chemical structure of KB-R9032

research to produce potent and highly water-soluble inhibitors was performed. In the series of studies of derivatives of some monocyclic aroyl guanidines, which have Na^+/H^+ exchange inhibitor properties, the structural requirement for a potent Na^+/H^+ exchange inhibitor has been gradually revealed. Some benzoxadine derivatives and their optical isomers and reactive subgroups in this class of compounds have been developed as a new series of agents with Na^+/H^+ exchange activities. Among these agents, KB-R9032 has been developed as a candidate that has sufficient potency and water-solubility (Fig. 5) as to enable it to be widely used as a therapeutic drug. KB-R9032 is exceedingly hydrophilic (solubility, approximately $27\,mg \cdot ml^{-1}$) and it is reported to have a strong inhibitory effect on Na^+/H^+ exchange, as evidenced by its IC_{50} (the concentration at which 50% inhibition is observed) [16,17] of $0.12\,\mu M$ for Na^+/H^+ exchange in a propionate swelling assay induced by sodium propionate. KB-R9032, the Na^+/H^+ exchange inhibitor, produced a significant inhibitory effect on reperfusion-induced arrhythmias in the current study, and it has been suggested that this drug may have the capability to mitigate the extent of cell injuries associated with ischemia-reperfusion. This study suggests that KB-R9032 has great potential as a useful therapeutic drug, and it is expected that more data on its effect on ischemia-reperfusion injuries will accumulate in clinical practice.

It was shown in this study that the Na^+/H^+ exchange inhibitor KB-R9032 suppressed reperfusion-induced arrhythmias. We propose that the mechanism of this effect is that significant suppression of Na^+/H^+ exchanges would have resumed after reperfusion, thereby preventing myocardial Ca^{2+} overload mediated by Na^+/Ca^{2+} exchange. KB-R9032 is a potent Na^+/H^+ exchange inhibitor and its highly water-soluble property is expected to be helpful in clinical practice.

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