Mechanism of Action of KRN2391 in Canine Coronary Vascular Bed

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ABSTRACT—The present studies were performed to clarify the mechanism of action of KRN2391 in various sizes of canine coronary artery. We used the responses of isolated large and small coronary arteries and the changes in coronary blood flow (CBF) as indicators reflecting the responses of conductive arteries and resistive arterioles, respectively. In isolated small coronary artery, the effect of KRN2391 (10^-8-10^-5 M) was antagonized by either methylene blue or glibenclamide. In isolated large coronary artery, the vasorelaxant effect of KRN2391 (10^-7-10^-4 M) and nicorandil (10^-7-10^-4 M) were antagonized by methylene blue (10^-7 M) but not by glibenclamide (10^-6 M). The relaxant effect of cromakalim was antagonized by glibenclamide but not by methylene blue in isolated large coronary artery. Intracoronary arterial injection of KRN2391, nicorandil or cromakalim produced an increase in CBF dose-dependently. Glibenclamide (5 mg/kg, i.v.) attenuated the increase in CBF caused by KRN2391, nicorandil and cromakalim. ED_{20}, the dose that produced an increase in CBF by 20 ml/min, increased about 5-fold for KRN2391 and nicorandil and about 12-fold for cromakalim after administration of glibenclamide. These results suggest that the mechanism of action of KRN2391 and nicorandil depends on the segment of coronary arteries; i.e., they show a nitrate action alone in large coronary artery, and a K-channel opening action in addition to a nitrate action as the size of the coronary artery decreases.

Keywords: KRN2391, Nicorandil, Cromakalim, Glibenclamide, Coronary artery

KRN2391, N-cyano-N'-(2-nitroxyethyl)-3-pyridinecarboximidamide monomethanesulfonate, showed potent vasodilating activity with selectivity for the coronary vascular bed in anesthetized dogs (1, 2). The vasodilating mechanism of KRN2391 is thought to be based on its dual mechanism of action as a nitrate and a K-channel opener because KRN2391-induced relaxation was inhibited by K-channel blockers and an inhibitor of soluble guanylate cyclase in isolated rat (3) and rabbit (4) aorta and isolated canine small coronary artery (5). Nicorandil is also reported to possess such a dual mechanism of action (6). In the coronary vascular bed, it has been demonstrated that the vasodilating action of nicorandil depends on the segment of coronary artery; i.e., nicorandil acts as a nitrate in large conductive coronary arteries (7) and as a K-channel opener in resistive coronary arterioles (8, 9). One of the authors of this paper reported that KRN2391 increased cyclic GMP formation in porcine large coronary artery (10), suggesting a nitrate-like action of KRN2391 in porcine coronary arteries. In experiments using anesthetized dogs, the increase in coronary blood flow induced by the intravenous administration of KRN2391 was also inhibited by glibenclamide, suggesting that K-channel opening action contributes to the dilating effect of KRN2391 on resistive coronary arteries (2). However, in this study, the role of the nitrate action of KRN2391 in its relaxant effect on resistive coronary arteries was not analyzed. In the present studies, we studied the contribution of the nitrate and K-channel opening actions of KRN2391 on various sizes of coronary artery compared with those of nicorandil and cromakalim.

MATERIALS AND METHODS

Isolated canine large and small coronary artery preparation

Left circumflex coronary arteries were obtained from beagle dogs of either sex weighing 8.8 - 13.0 kg killed with an over-dose of sodium pentobarbital (80 mg/kg, i.v.). The heart was then removed, and the coronary arteries (2.0 - 3.0 mm, O.D.) and the distal branch of the left circumflex coronary artery (0.4 - 0.8 mm, O.D.) were
dissected free from adhering connected tissue and used as large and small vessels, respectively. The arteries were cut into 2- to 3-mm-long ring segments, and the endothelium was removed by gentle rubbing. Removal of the endothelium was verified by the absence of a relaxant response to acetylcholine in preliminary experiments. Ring segments of the arteries were mounted on stainless steel wires in an organ bath filled with 10 ml Krebs-Henseleit solution of the following composition: 118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.2 mM KH2PO4, 25.0 mM NaHCO3 and 10.0 mM glucose, at 37°C aerated with a gas mixture of 95% O2 and 5% CO2. The artery rings were stretched by an initial resting tension of 1.5 g for the large coronary artery and 1.0 g for the small coronary artery. After an initial equilibration period of 120 min, the artery rings were contracted by changing the solution in the bath to one containing 25 mM K+, which was prepared by substituting NaCl with an equimolar amount of KCl. After the arteries reached a stable tension, vasodilators were added in a cumulative manner. Methylene blue (10^{-5} M) and glibenclamide (10^{-6} M) were added 20 min before the arteries were contracted by 25 mM KCl. Isometric tension was measured with a mechanical transducer (TB-611T; Nihon Kohden, Tokyo).

**Anesthetized dog preparation**

**General surgery:** Experiments were performed in 12 beagle dogs (9.3 – 12.5 kg) of either sex. The animals were initially anesthetized with 30 mg/kg sodium pentobarbital (i.v.) and then sodium pentobarbital was infused at a rate of 11 µg/kg/min via the left femoral vein to maintain anesthesia. Animals were artificially ventilated with room air using a respirator (SN-480-3; Shinano, Tokyo). Systemic blood pressure (SBP) was measured in the right femoral artery with a pressure transducer (TP-200T, Nihon Kohden) through a carrier amplifier (AP-641G, Nihon Kohden). Heart rate (HR) was measured with a heart rate counter (AT-601G, Nihon Kohden) triggered by the pressure pulse. The right femoral vein was cannulated for intravenous administration of glibenclamide. The chest of dogs was opened at the left 5th intercostal space. Coronary blood flow was measured with an electromagnetic flowmeter (MFV-2100, Nihon Kohden) using a probe placed around the circumflex branch of the left coronary artery. A polyethylene tube (O.D. 0.8 mm, I.D. 0.5 mm) was introduced into a distal branch of the circumflex coronary artery for intra-arterial (i.a.) injections of drugs. All parameters were recorded on a multi-channel thermorecorder (WT-687G, Nihon Kohden).

**Experimental protocol:** The experimental protocol consisted of two periods. The first period was the control phase; intra-arterial injections of KRN2391, nicorandil and cromakalim were performed to estimate their vasodilating effects 5 min after intravenous administration of 0.5 ml N,N'-dimethylformamide (DMF), the vehicle for glibenclamide. In the second period, the effects of KRN2391, nicorandil and cromakalim were observed from 5 min after intravenous administration of glibenclamide (5 mg/kg). The effect of nifedipine (0.1 µg/kg) was also examined in each period in all experiments. There were 3 groups; Group I consisted of 4 dogs who received doses of KRN2391 (0.01 – 1.0 µg/kg) by injection, in random order, at intervals of more than 5 min. Group II consisted of 4 dogs who received doses of nicorandil (0.1 – 30 µg/kg) by injection, in random order, at intervals of more than 5 min. Group III consisted of 4 dogs receiving doses of cromakalim (0.01 – 3.0 µg/kg) by injection, in random order, at intervals of more than 5 min. The duration and volume of injections were 15 sec and 0.23 ml.

**Drugs**

KRN2391, nicorandil and cromakalim were synthesized in our laboratory. Nifedipine and glibenclamide were purchased from Sigma Co. (St. Louis, MO, USA). Methylene blue was purchased from Wako Pure Chemicals (Osaka). For the in vitro experiments, KRN2391 was dissolved in Krebs-Henseleit solution at 10^{-2} M, nicorandil was dissolved in 0.1 N HCl at 5 x 10^{-2} M and cromakalim was dissolved in dimethylsulfoxide (DMSO) at 10^{-2} M. Glibenclamide and methylene blue were dissolved at 10^{-3} M in DMSO and at 10^{-2} M in double distilled water, respectively. These solutions were further diluted to the appropriate concentrations in Krebs-Henseleit solution. In the in vivo experiments, KRN2391 and nicorandil were dissolved in physiological saline, and cromakalim was dissolved in polyethylene glycol 200 / physiological saline (50 : 50 v/v). Nifedipine was dissolved in physiological saline / polyethylene glycol 400 / ethanol (70 : 15 : 15 v/v). Glibenclamide was dissolved in DMF at the appropriate concentration in 0.5 ml.

**Data analyses**

Data are presented as the mean ± S.E.M. In the in vitro experiments, the force of concentration produced by KCl was expressed in g, and relaxation caused by the test drugs was expressed as a percentage of the maximum relaxation obtained by addition of 10^{-4} M papaverine at the end of each experiment. In each preparation, the concentration-relaxation curve for the drug was fitted to a logistic equation:

\[ E = \frac{M \times A^p}{A^p + K^p} \]

where E is the normalized effect, M is the calculated maximum response, A is the concentration, K is the EC_{50} value and p is the slope parameter (11). EC_{50} values thus obtained were averaged for a number of arteries and ex-
pressed in terms of $-\log EC_{50}$. Differences were considered significant at a P value of less than 0.05 using analysis of variance for multiple comparisons. When multiple comparisons were made with a single control, Dunnett's test was used to determine significance. In the in vivo experiments, doses that produced an increase in coronary blood flow by 20 ml/min (approximately half-maximal effect, $ED_{20}$) were determined from the dose-response curves at three higher doses which were fitted by linear regression. Differences were evaluated, with significance taken as $P<0.05$, by the t-test for paired data for single comparisons.

RESULTS

Effect on isolated canine large coronary artery preparation

KRN2391 ($10^{-8}$–$10^{-5}$ M), nicorandil ($10^{-7}$–$10^{-4}$ M) and cromakalim ($10^{-7}$–$10^{-4}$ M) produced concentration-dependent relaxation in coronary arteries contracted by 25 mM KCl (Fig. 1). KRN2391 and nicorandil produced almost complete relaxation at their maximum-effect concentrations. However, cromakalim produced 86% relaxation at its maximum-effect concentration.

Glibenclamide and methylene blue had no effect on basal tension. The developed tension caused by 25 mM KCl was not significantly affected by methylene blue or glibenclamide (Fig. 1). The concentration-relaxation curves for KRN2391 and nicorandil shifted rightwards in the presence of $10^{-5}$ M methylene blue (Fig. 1, A and B), and the $EC_{50}$ values for KRN2391 and nicorandil in the presence of methylene blue were significantly greater than that for the control (Table 1). In contrast, the concentration-relaxation curve for cromakalim in the presence of methylene blue was not significantly different from that

Table 1. Effects of KRN2391, nicorandil and cromakalim on maximum effect, $EC_{50}$ and slope power in isolated canine large coronary artery

| Treatment            | Maximum effect (%) | $EC_{50}$ value ($-\log M$) | Slope power |
|----------------------|--------------------|------------------------------|-------------|
| KRN2391              |                    |                              |             |
| 25 mM KCl            | 98.00±0.91         | 6.76±0.09                    | 1.64±0.12   |
| 25 mM KCl + MB       | 90.15±3.50*        | 6.54±0.04*                   | 0.98±0.08   |
| 25 mM KCl + GBC      | 92.34±2.00         | 6.91±0.08                    | 2.44±0.38*  |
| Nicorandil           |                    |                              |             |
| 25 mM KCl            | 94.34±0.80         | 1.10±0.05                    | 1.37±0.07   |
| 25 mM KCl + MB       | 1000              | 5.11±0.06*                   | 0.80±0.03*  |
| 25 mM KCl + GBC      | 91.93±0.76         | 6.12±0.06                    | 1.58±0.13   |
| Cromakalim           |                    |                              |             |
| 25 mM KCl            | 80.36±4.36         | 6.23±0.10                    | 1.85±0.17   |
| 25 mM KCl + MB       | 90.31±1.69*        | 6.20±0.14                    | 1.37±0.20   |
| 25 mM KCl + GBC      | 82.90±4.55         | 5.15±0.19*                   | 2.71±0.86   |

The concentration-relaxation curves for KRN2391, nicorandil and cromakalim were analyzed by computer fitting to the logistic equation described in the Methods. *Since the curve for nicorandil in the presence of methylene blue was not shown as a complete sigmoid curve, the maximum effect was fixed at 100%. MB, Methylene blue; GBC, Glibenclamide. $P<0.05$, significantly different from 25 mM KCl values. Data are represented as the mean±S.E.M. of 6–8 experiments.
Glibenclamide (10⁻⁶ M) did not influence the concentration-relaxation curves of KRN2391 and nicorandil, and the concentration-relaxation curve for cromakalim in the presence of glibenclamide shifted to the right (Fig. 1C). The EC₅₀ value for cromakalim in the presence of glibenclamide was significantly greater than that for the control (Table 1).

**Effect on isolated canine small coronary artery preparation**

KRN2391 (10⁻⁸–10⁻⁴ M) produced concentration-dependent relaxation in coronary arteries contracted by 25 mM KCl (Fig. 2). KRN2391 produced almost complete relaxation at their maximum-effect concentrations.

Glibenclamide and methylene blue had no effect on basal tension. The developed tension caused by 25 mM KCl was not significantly affected by methylene blue or glibenclamide (Fig. 2). The concentration-relaxation curves for KRN2391 shifted rightwards in the presence of 10⁻⁵ M methylene blue or 10⁻⁶ M glibenclamide (Fig. 2), and the EC₅₀ values for KRN2391 in the presence of methylene blue or glibenclamide were significantly greater than that for the control (Table 2).

**Effect on coronary blood flow**

The administration of glibenclamide (5 mg/kg, i.v.) produced a slight increase in mean blood pressure (MBP) from 108.8 ± 6.1 to 126.8 ± 7.1 mmHg (n = 12, P < 0.01) and a decrease in HR from 152.7 ± 12.1 to 143.7 ± 12.9 beats/min (n = 12, P < 0.01). The basal CBF was also decreased by glibenclamide (Table 3).

### Table 2. Effects of KRN2391 on maximum effects, EC₅₀ and slope power in isolated canine small coronary artery

| Drug          | Maximum effect (%) | EC₅₀ value (log M) | Slope power |
|---------------|--------------------|--------------------|-------------|
| KRN2391       |                    |                    |             |
| 25 mM KCl     | 96.43 ± 0.81       | 6.82 ± 0.14        | 1.93 ± 0.56 |
| 25 mM KCl + MB| 86.52 ± 2.69*      | 6.02 ± 0.08*       | 1.41 ± 0.19 |
| 25 mM KCl + GBC| 92.31 ± 1.30      | 6.37 ± 0.19*       | 1.93 ± 0.34 |

The concentration-relaxation curves for KRN2391, nicorandil and cromakalim were analyzed by computer fitting to the logistic equation described in the Methods. MB, Methylene blue; GBC, Glibenclamide. *P < 0.05, significantly different from 25 mM KCl values. Data are represent the mean ± S.E.M. of 6 experiments.

### Table 3. Effect of glibenclamide on basal values of coronary blood flow (CBF)

| Drug (µg/kg, i.a.) | CBF (ml/min) | Nicorandil | Nifedipine |
|--------------------|--------------|------------|------------|
| Control            | 16 ± 2       | 16 ± 2     | 16 ± 2     |
| Glibenclamide      | 17 ± 1       | 16 ± 1     | 16 ± 1     |

Values are the mean ± S.E.M. of 4 experiments. *P < 0.05, significant difference from the corresponding control value.
Intra-arterial injection of KRN2391 (0.01–0.3 μg/kg, n=4), cromakalim (0.01–1.0 μg/kg, n=4) and nicorandil (0.1–10 μg/kg, n=4) produced dose-dependent increases in CBF (Fig. 3). The order of potency shown by the ED<sub>20</sub> value was KRN2391 > cromakalim > nicorandil (Table 4). SBP was not essentially changed by these drugs but was slightly decreased by 10 μg/kg nicorandil.

The dose-response curves for KRN2391, nicorandil and cromakalim before and after the administration of glibenclamide are shown in Fig. 3. After i.v. administration of glibenclamide (5 mg/kg), the dose-response curves for KRN2391, nicorandil and cromakalim shifted to the right. To evaluate the antagonistic effect of glibenclamide, ED<sub>20</sub> values were calculated from each dose-response curve for each vasodilator before and after the administration of glibenclamide (Table 4). The ED<sub>20</sub> values for KRN2391, nicorandil and cromakalim were significantly increased by glibenclamide, and the increase in ED<sub>20</sub> value for cromakalim was greater than those for KRN2391 and nicorandil. Glibenclamide had no effect on the increase in blood flow caused by nifedipine (0.1 μg/kg) in each group (Fig. 3).

The doses that produced an increase in coronary blood flow by 20 ml/min (approximately half-maximal effect, ED<sub>50</sub>) were determined from the dose-response curves at three higher doses which were fitted by linear regression. Values are the mean ± S.E.M. *P < 0.05, significant difference from the corresponding control value.

Table 4. Effect of glibenclamide on the coronary vasodilating effects of KRN2391, nicorandil and cromakalim

| Group              | ED<sub>20</sub> (μg/kg, i.a.) |
|--------------------|-------------------------------|
|                    | KRN2391 | Nicorandil | Cromakalim |
| Control            | 0.08±0.01 | 3.24±0.92 | 0.17±0.02 |
| Glibenclamide      | 0.41±0.04* | 17.58±5.11* | 1.90±0.74* |

DISCUSSION

In canine coronary artery, the mechanism of action of nicorandil is thought to depend on the segment of artery (7, 9, 12). KRN2391, possessing nitrate and K-channel opening actions like nicorandil, is also thought to show a different mechanism of action depending on the segment of artery. Actually, KRN2391 as well as nicorandil is suggested to show a K-channel opening action in resistive coronary arterioles of dogs because the increases in coronary blood flow induced by intravenous administration of KRN2391 and nicorandil were inhibited by glibenclamide (2). In addition, Jinno et al. (10) reported that KRN2391 produced an increase in cyclic GMP formation in porcine large coronary arteries, suggesting the nitrate action of KRN2391. Therefore, it is considered that KRN2391 and nicorandil show a K-channel opening action in addition to a nitrate action in proportion to the size of coronary artery. In the present study, we performed a further examination to more closely analyze the mechanism of action of KRN2391 according to the size of coronary artery us-
ing isolated canine large and small coronary arteries and anesthetized dogs. The responses of isolated large and small coronary arteries and the changes of CBF were used as indices reflecting the responses of conductive arteries and resistive arterioles, respectively.

The mechanism of vasodilation of KRN2391 and nicorandil were different from that of cromakalim in isolated canine large coronary artery. KRN2391- and nicorandil-induced relaxation were antagonized by methylene blue, an inhibitor of soluble guanylate cyclase (13), but not by glibenclamide, a pharmacologic inhibitor of K-channels (14–16). In contrast, cromakalim-induced relaxation was antagonized by glibenclamide but not by methylene blue. These results suggest that KRN2391 and nicorandil predominantly act as a nitrate and cromakalim as a K-channel opener in large segments of coronary artery. These results with nicorandil and cromakalim are also in agreement with the report from Satoh et al. (7). Recently, we found that the relaxant effect of KRN2391 on large coronary arteries of pigs was antagonized by oxyhemoglobin, a pharmacological antagonist of nitrovasodilators, and glibenclamide, but that of nicorandil was antagonized by oxyhemoglobin alone (17). The discrepancy in the mechanism of action between canine and porcine large coronary arteries may be due to the species differences. In isolated canine small coronary artery, KRN2391-induced relaxation was antagonized by either methylene blue or glibenclamide. Furthermore, Okada et al. (5) reported in detail that KRN2391 possessed a dual mechanism of action by examining its effects on both intracellular calcium concentration and force in canine small coronary artery. These findings in isolated small coronary artery are different from the present results which were obtained in isolated canine large coronary artery. These results suggest that KRN2391 act as nitrates and K-channel openers in a small segment of coronary artery. Thus, the mechanism of action of KRN2391 appear to depend on the segment of coronary artery.

Stimulation of guanylate cyclase by nicorandil is supported by earlier studies. Endoh and Taira (18) reported that nicorandil increased cyclic GMP formation in canine mesenteric arteries, and this increase was also observed by Kukovetz et al. (19). In contrast, Coldwell and Howlett (20) showed that cromakalim had no appreciable effect of cyclic GMP formation in rabbit mesenteric arteries. These results appear to support the present study in which methylene blue did not affect cromakalim-induced relaxation.

In this study, KRN2391 and nicorandil produced almost complete relaxation of isolated coronary artery contracted by 25 mM KCl. However, cromakalim produced insufficient relaxation even at a concentration showing maximum effect. The present results with KRN2391, nicorandil and cromakalim are in good agreement with the previous results using the same preparation (5, 17, 21). This insufficient relaxation by cromakalim seemed to be based on the finding that the relaxant effect of a K-channel opener in vascular smooth muscle contracted by KCl depends on the concentration of KCl (21). Therefore, these results may also reflect the fact that a nitrate action of KRN2391 and nicorandil contribute to their relaxant effects in large and small segments of coronary arteries.

In the in vivo experiments, we examined the effects of glibenclamide on the increase in CBF induced by KRN2391, nicorandil and cromakalim to clarify their mechanisms of action in resistive coronary arterioles. Glibenclamide attenuated the increase in CBF induced by intra-arterial administration of KRN2391, nicorandil and cromakalim. In contrast, glibenclamide had no effect on the increase in CBF induced by nifedipine. Therefore, it is considered that a K-channel opening action contributed to the mechanisms of action of KRN2391, nicorandil and cromakalim in increasing CBF; i.e., these drugs act as K-channel openers in resistive coronary arterioles. Kingsbury et al. (22) and Yoneyama et al. (8), respectively, reported that glibenclamide inhibited increases in coronary blood flow induced by KRN2391 and nicorandil in isolated blood-perfused canine heart preparation. Furthermore, it has been reported that the increases in coronary blood flow induced by intravenous administration of KRN2391 and nicorandil are inhibited by glibenclamide. However, when the inhibitory effects of glibenclamide were estimated in terms of change in ED₅₀ for KRN2391, nicorandil and cromakalim, the magnitude of the shift in the dose-response curve induced by glibenclamide was about 11-fold for cromakalim compared to about 5-fold for KRN2391 and nicorandil. Imagawa et al. (9) also reported that the increase in CBF induced by the intra-arterial injection of nicorandil was inhibited by glibenclamide in anesthetized dogs and the magnitude of the shift in the dose-response curve caused by glibenclamide was greater for cromakalim than for nicorandil. These observations were similar to the present results with nicorandil and cromakalim. In addition, they observed that glibenclamide had no effect on the increase in CBF induced by nitroglycerin (9). Thus, the other action in addition to the K-channel opening action of KRN2391 and nicorandil may partly contribute to their vasodilating effect in resistive coronary arterioles; i.e., they are assumed to possess a nitrate action. The characteristics of the drugs that possess both nitrate and K-channel opening actions have been extensively reviewed (23).

The intravenous administration of glibenclamide slightly altered the basal values of mean blood pressure and coronary blood flow. This result indicates that ATP-sensi-
tive K-channels may partly contribute to maintain the basal cardiovascular condition.

In summary, the present results show that the mechanisms of action of KRN2391 depend on the segment of coronary artery; i.e., KRN2391 shows a nitrate action alone in canine large conductive arteries and a K-channel opening action in addition to a nitrate action as the size of the coronary artery decrease.

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