An analysis of the nitrate-like and K channel opening actions of KRN2391 in canine coronary arterial smooth muscle

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1 To clarify the mechanism of action of KRN2391, a new vasodilator containing a nitroxy group, its effects on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and force of contraction in canine coronary artery were compared with those of two derivatives lacking the nitro group.

2 KRN2391, its hydroxy and acetoxy derivative (Compound 2 and Compound 3, respectively) partially reduced $[\text{Ca}^{2+}]_i$ in 5 or 30 mM KCl physiological salt solution (PSS), effects which were antagonized by glibenclamide. No KRN2391-induced change in $[\text{Ca}^{2+}]_i$ was observed in 90 mM KCl-PSS.

3 The order of potency in reducing $[\text{Ca}^{2+}]_i$ and inhibiting the contracture in 30 mM KCl-PSS was: KRN2391 > Compound 3 > Compound 2.

4 In 30 mM KCl-PSS, KRN2391 shifted the $[\text{Ca}^{2+}]_i$ -force relationship so that a greater increase in $[\text{Ca}^{2+}]_i$ was needed to produce force. Compounds 2 and 3 were ineffective. The $[\text{Ca}^{2+}]_i$ -force curve obtained in 90 mM KCl-PSS was shifted to the right by KRN2391 (10^{-4} M).

5 The ability of KRN2391 to reduce the force of contraction in both 30 mM and 90 mM KCl-PSS was inhibited by 10^{-5} M methylene blue.

6 KRN2391 inhibited U46619-induced contractions; this effect was associated with a reduction of $[\text{Ca}^{2+}]_i$, which decreased below the basal level.

7 Thus, KRN2391 is a potent vasodilator in canine coronary artery. It possesses nitrate-like and potassium channel opening actions and can be designated as a nitrate-potassium channel opener (N-K) hybrid. The denitrate derivatives of KRN2391 are specific K channel openers. The nitroxy moiety in KRN2391 is important for not only its action as a nitrate but also its potency as a K channel opener.

Keywords: N-K hybrid; K channel opener; nitrate; KRN2391; glibenclamide; fura-2; intracellular calcium concentration; relaxation; dog coronary artery

Introduction

A hybrid nitrate and potassium (K) channel activating (N-K hybrid) mechanism was proposed by Taira (1989), as the mechanism of action of nicorandil which has the dual vasodilator mechanism of a nitrate (Holzmann, 1983; Endoh & Taira, 1983) and a K channel opener (Karashima *et al.*, 1982; Weir & Weston, 1986; see reviews, Taira, 1987; Edwards & Weston, 1990). Nicorandil is the only N-K hybrid that is clinically used for the treatment of patients with angina pectoris at present. Recently, it has been reported that KRN2391, N-cyano-N'-(2-nitroxyethyl)-3-pyridinecarboximidamide methanesulphonate (Figure 1), has vasorelaxant and antihypertensive effects (Kashiwabara *et al.*, 1990). The vasodilator effect of KRN2391 was partly inhibited by K channel

blockers, e.g., glibenclamide, tetraethylammonium and 4-aminopyridine, and also by methylene blue and oxyhaemoglobin (Kashiwabara *et al.*, 1991). KRN2391 also increased ^{86}Rb efflux from rat aorta. Nicorandil possesses both K channel opening and nitrate-like actions in canine coronary arteries (Yanagisawa *et al.*, 1990) which suggests that relaxations produced by KRN2391 could also be associated with a similar dual mechanism (Kashiwabara *et al.*, 1991).

Yanagisawa *et al.* (1989, 1990) found that the effects of K channel openers and nitrates could be clearly characterized by simultaneous measurement of intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) measured by fura-2 (Grynkiewicz *et al.*, 1985) and force of contraction in canine coronary arteries. K channel openers, like cromakalim, reduce the $[\text{Ca}^{2+}]_i$ due to the closure of voltage-dependent Ca^{2+} channels by hyperpolarization (Weston, 1989; Nelson *et al.*, 1990) without affecting the Ca^{2+} sensitivity of contractile proteins (Yanagisawa *et al.*, 1990). In contrast, nitrates, like nitroglycerin, relax the contracture produced by KCl by reducing the sensitivity of contractile proteins to Ca^{2+} but without affecting $[\text{Ca}^{2+}]_i$ (Yanagisawa *et al.*, 1989).

The present study was performed to clarify the mechanisms of vasodilatation of KRN2391 by examining its effects on both $[\text{Ca}^{2+}]_i$ and force in canine coronary arteries. Since KRN2391 has a nitroxy moiety in its structure (Figure 1), we determined whether this substituent plays an important role in its pharmacological activity by comparing its effects with those of two derivatives lacking the nitro group (denitrate derivatives), Compound 2 (Comp. 2, Ki3315) and Compound 3 (Comp. 3, Ki4032).

Methods

Preparation of arterial rings

Hearts were obtained from mongrel dogs of either sex, weighing 5 to 15 kg, anaesthetized with pentobarbitone sodium

Figure 1 Chemical structures of KRN, Compound 2 (Ki3315) and Compound 3 (Ki4032). * The free bases of KRN2391 and Comp. 2 were used throughout these experiments.

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(30 mg kg⁻¹, i.v.). Coronary arterial rings (0.7–1.2 mm in diameter, about 1 mm in width) were dissected and connective tissues were carefully removed in a dissecting chamber under a binocular microscope (SZ, Olympus, Tokyo, Japan). The endothelium was removed by gentle rubbing with forceps and the effectiveness of removal was checked by the abolition of acetylcholine-induced relaxation. The luminal side of the arterial ring was turned to face outwards.

Fluorescence measurements

We performed fluorescence measurements as previously described (Yanagisawa *et al.*, 1989). In brief, coronary arterial rings were exposed to 10 μ M fura-2 acetoxymethyl ester (fura-2 AM) for about 2 h at 37°C. The non-cytotoxic detergent, pluronic F-127 (0.1% w/v), was premixed in the loading physiological salt solution (PSS) to help dissolve the fura-2 AM in PSS. After the fura-2 loading, muscles were rinsed with normal PSS for more than 1 h and then used for experiments. Fluorescence was measured in a fluorimeter equipped with a dual wavelength excitation device (CAM-200 or CAM-220, Japan Spectroscopic, Tokyo, Japan) connected to an inverted microscope (TMD, Nikon, Tokyo, Japan). The fluorescence image was obtained by focusing on the smooth muscle cells in the medial layer with a Nikon CF u.v. (Fluor) 10 $\times$ objective lens. The muscle ring was placed horizontally in a temperature-controlled 0.4 ml tissue bath which was mounted on the inverted microscope and perfused with PSS at a rate of 4 ml min⁻¹. The muscle ring was stretched to a resting tension of about 5 mN between two tungsten needles, one of which was glued to a transducer element (AE801, AME, Horten, Norway or U-10230, Shinko, Tokyo, Japan). The photosignals and the mechanical activity were measured simultaneously and both were recorded on a chart recorder (Recti-horiz-8k, NEC-San-ei, Tokyo, Japan). They were also digitized by A/D converters and fed into a microcomputer (PC-9801, NEC, Tokyo, Japan) for further calculation and graphical analysis (Himpens & Somlyo, 1988). Every experiment started with perfusion of 90 mM KCl-PSS for 10 min. At the end of each experiment, the cell membrane was lysed with a detergent, Triton X-100 (1%), and autofluorescence signals were determined by quenching fura-2 signals with MnCl₂. We subtracted the F₃₄₀ and F₃₈₀ values due to autofluorescence from the corresponding values of F₃₄₀ and F₃₈₀ obtained under conditions of fura-2 loading to derive a recalculated ratio, r_c . Since it is difficult to calculate the absolute concentration of [Ca²⁺]_i (Konishi *et al.*, 1988; Karaki, 1989), we used the relative F₃₄₀/F₃₈₀ ratio as an indicator of [Ca²⁺]_i (Sato *et al.*, 1988; Yanagisawa *et al.*, 1989). Changes in the ratio, r_c (changes in [Ca²⁺]_i) and those in force were expressed as percentages of the differences between basal values and those obtained with a 10 min perfusion with 90 mM KCl-PSS. None of the drugs and chemicals used in the present study, with the exception of fura-2, MnCl₂ and methylene blue affected fluorescence signals at the concentrations used.

Drugs and solutions

The composition (mM) of normal or 5 mM KCl physiological salt solution (PSS) was as follows: NaCl 140, KCl 5, CaCl₂ 2.5, MgCl₂ 2.5, glucose 11.1, HEPES 3 (pH 7.4). The solution was equilibrated with 100% O₂ at 37°C. High KCl-PSS was made by substituting NaCl with equimolar KCl. Ca²⁺-free PSS was made by removing CaCl₂ from normal PSS and sometimes by adding 1 mM EGTA to the solution.

Drugs and chemicals were obtained from the following sources: KRN2391, Comp. 2 and Comp. 3 (Kirin Brewery, Tokyo, Japan), glibenclamide (Yamanouchi, Tokyo, Japan), methylene blue trihydrate (Kanto Chemical, Tokyo, Japan), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α} ; Sigma, St. Louis, MO, U.S.A.), EGTA (glycol-etherdiamine-tetraacetic acid), HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid), fura-2 AM and pluronic

ic F-127 (Dojin, Kumamoto, Japan), DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). The free bases of KRN2391 and Comp. 2 were used throughout the experiments. KRN2391, Comp. 2 and Comp. 3 were diluted with PSS immediately before use and the pH of the solution was adjusted to 7.4. Fura-2 AM was dissolved in DMSO at a concentration of 1 mM. Pluronic F-127 was dissolved in 25% w/v in DMSO. Glibenclamide was dissolved in DMSO to give a concentration of 10 mM. U46619 was dissolved in ethanol to give a concentration of 1 mM. Other drugs or chemicals were dissolved in distilled water and diluted to the desired concentrations by PSS.

Analysis of concentration-effect curves

The concentration-effect curves for drugs in reducing the force of contraction, and the increased [Ca²⁺]_i produced by high KCl or U46619, were expressed as a % reduction from the pre-drug values, and computer fitted to a logistic equation:

$$E = 100 - M \times A^p / (A^p + K^p) \quad (1)$$

where E is the normalized effect, M is the maximum effect, A is the drug concentration, K is the EC₅₀ value of each drug and p is the slope parameter. When the maximum effect of each drug was not obtainable because of its limited solubility, M was fixed at 100. The concentration-effect curves for KRN2391 in reducing [Ca²⁺]_i below the basal level in 5 mM KCl-PSS were also computer fitted to equation (1). The maximum effect, M, however, was the maximum reduction of [Ca²⁺]_i produced on perfusion with Ca²⁺-free PSS containing 1 mM EGTA which was taken as 100%. The concentration-effect curves for extracellular Ca²⁺ concentration ([Ca²⁺]_o) were also computer fitted to a logistic equation:

$$E = M \times A^p / (A^p + K^p) \quad (2)$$

where E is the effect normalized by the differences between basal values and those of 90 mM KCl at 10 min, A is [Ca²⁺]_o, K is EC₅₀ value of [Ca²⁺]_o and p is the slope parameter. EC₅₀ values were presented as pD₂ (pD₂ = -log EC₅₀). Experimental values are given as mean $\pm$ s.e.mean. Statistical significance of results was evaluated by a one-way analysis of variance followed by Dunnett multiple-comparison test or Kruskal-Wallis test followed by Student's multiple-comparison test after Bartlett's test for pre-analysis of variance. A P value smaller than 0.05 was considered to be significant.

Results

Effect of KRN2391 on [Ca²⁺]_i and KCl-induced contractures

In canine coronary arteries loaded with fura-2, KRN2391 (10⁻⁸–10⁻⁴ M) reduced the [Ca²⁺]_i below the based level in 5 mM KCl-PSS, without affecting the resting force (Figure 2a). The pD₂ value for this effect was 6.79 $\pm$ 0.10 (n = 6) (Table 1). Perfusion with 30 mM KCl-PSS increased [Ca²⁺]_i and elicited a contracture. KRN2391 (3 $\times$ 10⁻⁸–10⁻⁴ M) reduced both [Ca²⁺]_i and contracture in a concentration-dependent manner (Figure 2b). The contracture was nearly abolished by 10⁻⁴ M KRN2391 but the increased [Ca²⁺]_i was only partially inhibited. The effects of KRN2391 could be reversed by washing. The summarized data from five muscles are shown in Figure 2c, and the results of the analysis of the concentration-effect curves for KRN2391 to reduce the increased [Ca²⁺]_i and contracture produced by 30 mM KCl-PSS are summarized in Table 1. Thus, although the pD₂ value for inhibition of contracture (6.06) was slightly but not significantly smaller than that associated with a reduction of the increase in [Ca²⁺]_i (6.25), the maximum inhibitory effect on force (91%) was much larger than on [Ca²⁺]_i (39%) (Table 1). The ability

Figure 2 Effects of KRN2391 on the increase in $[Ca^{2+}]_i$ and force of contraction produced by high KCl-PSS in canine coronary arteries. (a) Typical traces of the force and $[Ca^{2+}]_i$ (indicated as fluorescence ratio) under resting conditions (5 mM KCl-PSS). The concentrations of KRN2391 (10^{-8} – 10^{-4} M) were increased cumulatively. (b) Typical traces of the force and $[Ca^{2+}]_i$ in 30 mM KCl-PSS. (c) The summarized data obtained from six preparations in 30 mM KCl-PSS. The force of contraction (○) and the increase in $[Ca^{2+}]_i$ (●) are expressed as a percentage of each effect to 90 mM KCl-PSS at 10 min. The force of contraction and the difference in the ratio_{re} in the presence of 90 mM KCl-PSS at 10 min were 9.9 ± 0.7 mN and 0.21 ± 0.05 , respectively. Vertical bars show s.e.mean. (d) Effects of KRN2391 (3×10^{-7} – 10^{-4} M) on the force of contraction (○) and increase in $[Ca^{2+}]_i$ (●) in 90 mM KCl-PSS. Data are expressed as mean ($n = 6$); s.e.mean shown by vertical bars.

Table 1 Effects of KRN2391 and its denitrate derivatives on the $[Ca^{2+}]_i$ and force of contraction

	n	Maximum effect (%)	Reduction of $[Ca^{2+}]_i$ pD_2	p	Maximum effect (%)	Reduction of force pD_2	p
KRN2391							
5 mM KCl	6	66.1 ± 5.0	6.79 ± 0.10	1.11 ± 0.16	No effect (<4)		
30 mM KCl	5	39.1 ± 5.6	6.25 ± 0.11	1.23 ± 0.11	91.4 ± 3.2	6.06 ± 0.13	1.37 ± 0.24
30 mM KCl + Glib	6	25.8 ± 6.0	$4.61 \pm 0.17^{**}$	1.31 ± 0.26	88.9 ± 6.0	$4.92 \pm 0.28^{**}$	$0.83 \pm 0.06^*$
30 mM KCl + MB	6	37.5 ± 4.6	6.16 ± 0.14	1.46 ± 0.24	86.1 ± 5.4	5.54 ± 0.14	$0.92 \pm 0.05^*$
90 mM KCl	6		no effect (<4)		100*	4.25 ± 0.16	0.64 ± 0.06
90 mM KCl + MB	5		no effect (<4)		—	<4	—
U46619 (3×10^{-7} M)	5	145.7 ± 8.0	6.58 ± 0.08	1.72 ± 0.18	91.7 ± 1.2	6.45 ± 0.04	1.52 ± 0.19
Compound 3							
30 mM KCl	5	55.3 ± 9.2	$5.22 \pm 0.77^{**}$	1.21 ± 0.11	$70.4 \pm 4.5^{**}$	$4.89 \pm 0.07^{**}$	1.49 ± 0.14
30 mM KCl + Glib	6	—	<3.52	—	—	<3.52	—
90 mM KCl	6		No effect (<3.52)			No effect (<3.52)	
Compound 2							
30 mM KCl	5	—	<3	—	—	<3	—

The concentration-effect curves for KRN2391, Compound 3 and Compound 2 were analysed by computer fitting to the logistic equation (1) described in the methods. $pD_2 = -\log EC_{50}$ and p is the slope parameter. * Maximum effect was fixed at 100. Glib and MB are glibenclamide (10^{-5} M) and methylene blue (10^{-5} M), respectively. —: Not determined. Data show mean $\pm$ s.e.mean. Significantly different from 30 mM KCl values: * $P < 0.05$; ** $P < 0.01$.

of KRN2391 to reduce $[Ca^{2+}]_i$ was dependent on the concentration of KCl. In 90 mM KCl-PSS, although the increased $[Ca^{2+}]_i$ was not changed by KRN2391, it inhibited the force of contraction in a concentration-dependent manner (Figure 2d). The relaxant potency of KRN2391 was also dependent on KCl concentration, i.e., the greater the depolarization of muscle, the lower the pD_2 values (Table 1). These results indicate that KRN2391 has a dual vasodilator mechanism of action, i.e., reduction of $[Ca^{2+}]_i$ and an inhibition of contracture independent of a reduction of the increased $[Ca^{2+}]_i$.

Effects of the denitrate derivatives of KRN2391 on KCl-induced increase in $[Ca^{2+}]_i$ and contractures

In the presence of 30 mM KCl-PSS, Comp. 3 (10^{-7} – 3×10^{-4} M) reduced both $[Ca^{2+}]_i$ and contracture in a concentration-dependent manner (Figure 3), with pD_2 values of 5.22 ± 0.07 and 4.89 ± 0.07 , respectively. Since the concentration-response curves did not reach maximal effects, the calculated values in Table 1 are the approximations of true values for each compound. Although the maximum inhibitory effect of Comp. 3 on $[Ca^{2+}]_i$ was slightly larger than that of KRN2391, the maximum mechano-inhibitory effect of Comp. 3 was less than that of KRN2391 (Table 1). Comp. 2 (3×10^{-5} – 10^{-3} M) slightly reduced both the increase in $[Ca^{2+}]_i$ and contracture induced by 30 mM KCl-PSS (Figure 3). When arterial rings were perfused with 90 mM KCl-

Figure 3 Effects of KRN2391 (●), Compound 3 (▲) and Compound 2 (■) on the increase in $[Ca^{2+}]_i$ (a) and force of contraction (b) produced by 30 mM KCl. The $[Ca^{2+}]_i$ and force are expressed as a percentage of each pre-drug value. The pre-drug value of $[Ca^{2+}]_i$ and force expressed as a percentage of the effect to 90 mM KCl-PSS at 10 min was 54.1 ± 3.4 and $29.0 \pm 1.9\%$ (KRN2391), 51.1 ± 4.2 and $28.1 \pm 5.1\%$ (Compound 3) and 48.9 ± 2.5 and $19.3 \pm 2.4\%$ (Compound 2), respectively. Data are expressed as mean ($n = 5$); s.e.mean shown by vertical bars.

PSS, Comp. 3 had no effect on either the force of contraction or on the increased $[Ca^{2+}]_i$ (data not shown).

Modification by glibenclamide of the effects of KRN2391 and its denitrate derivatives

Figure 4 shows the influence of 10^{-5} M glibenclamide on the ability of KRN2391 and Comp. 3 to reduce increased $[Ca^{2+}]_i$ and force of contraction produced by 30 mM KCl-PSS. Although glibenclamide (10^{-5} M) did not significantly change $[Ca^{2+}]_i$ or the contracture induced by 30 mM KCl, it antagonized the effect of KRN2391 (10^{-7} – 10^{-4} M) on these parameters (Figure 4a). The ability of KRN2391 to reduce $[Ca^{2+}]_i$ was more effectively inhibited by glibenclamide than the inhibition of force of contraction (Figure 4a; Table 1). The influence of glibenclamide (10^{-5} M) on the effects of Comp. 3 is shown in Figure 4b. The rightward shift by glibenclamide was almost the same for both the $[Ca^{2+}]_i$ and the force of contraction. Although glibenclamide similarly antagonized the reduction of $[Ca^{2+}]_i$ produced by both KRN2391 and Comp. 3, it more effectively antagonized the reduction of contracture caused by Comp. 3 than that by KRN2391 (Figure 4, Table 1). The ability of Comp. 2 to reduce $[Ca^{2+}]_i$ and contracture in 30 mM KCl-PSS was also inhibited by glibenclamide; $[Ca^{2+}]_i$ and force (%) were 81.0 ± 2.7 and $70.4 \pm 9.8\%$ in the presence of Comp. 2 (10^{-3} M) alone, but 96.8 ± 2.5 and $100.5 \pm 4.1\%$ in the presence of both Comp. 2 and glibenclamide (10^{-5} M), respectively ($P < 0.01$ for the changes in both $[Ca^{2+}]_i$ and force; $n = 5$).

Effect of KRN2391 or its denitrate derivatives on the relationship between $[Ca^{2+}]_i$ and force in 30 mM KCl-depolarized artery

Figure 5 shows the relationship between $[Ca^{2+}]_i$ and force in the absence (control) and the presence of KRN2391 (3×10^{-8} – 10^{-4} M), KRN2391 plus glibenclamide, Comp. 3 (10^{-7} – 3×10^{-4} M) and Comp. 2 (3×10^{-5} – 10^{-3} M) in 30 mM KCl-depolarized canine coronary arteries. The relationship was almost linear in the presence or absence of KRN2391 in 30 mM KCl-PSS. The line for KRN2391 was steeper than that of the control, and in the presence of glibenclamide it was much steeper than in both the control and KRN2391 alone groups (Figure 5a). In contrast, the slopes in the presence of Comp. 2 and 3 were almost the same as that of the control. These results show that, in addition to its ability (glibenclamide-sensitive) to reduce $[Ca^{2+}]_i$, KRN2391 also reduces the sensitivity of contractile proteins to Ca^{2+} in 90 mM KCl-PSS (Figure 3) and even in 30 mM KCl-PSS. In contrast, the relaxant effects of Comp. 2 and 3 are due to a decrease in $[Ca^{2+}]_i$, and not to a change in Ca^{2+} sensitivity of the contractile proteins.

Effect of KRN2391 on extracellular Ca^{2+} -induced changes in $[Ca^{2+}]_i$ and mechanical activity in 90 mM KCl-depolarized artery

As previously described, the relaxant potency of KRN2391 was dependent on the KCl concentration (Table 1) and furthermore KRN2391 was able to exert vasorelaxant effects without a detectable reduction in $[Ca^{2+}]_i$. To gain an insight into this mechanism, the effects of KRN2391 were examined under conditions in which $[Ca^{2+}]_i$ was changed without alteration of the membrane potential. A segment of artery was at first perfused with Ca^{2+} -free PSS for 10 min and then with Ca^{2+} -free 90 mM KCl-PSS. Removal of extracellular Ca^{2+} reduced $[Ca^{2+}]_i$ below the basal level ($-20.7 \pm 1.6\%$; expressed as a percentage of the increase following 10 min exposure to 90 mM KCl-PSS), whereas the basal force was not changed. Subsequent exposure of the muscle to 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ ($-20.4 \pm 3.8\%$) or any change in force. During continued exposure to 90 mM

Figure 4 Influence of glibenclamide on the KRN2391- and Compound 3-induced reduction of $[Ca^{2+}]_i$ and contraction produced by 30 mM KCl-PSS. Effects of KRN2391 (a) and Compound 3 (b) in the absence (○) and the presence (●) of 10^{-5} M glibenclamide. Glibenclamide was added 10 min after treatment with 30 mM KCl-PSS and KRN2391 was added 20 min after the addition of glibenclamide. The $[Ca^{2+}]_i$ and force are expressed as a percentage of each pre-drug value. $[Ca^{2+}]_i$ and force (%) were 61.9 ± 2.4 and $16.5 \pm 3.3\%$ before the addition of glibenclamide, and 54.7 ± 2.7 and $12.1 \pm 1.6\%$ after the addition of glibenclamide, respectively. Data are expressed as mean ($n = 5$); s.e.mean shown by vertical bars.

KCl-PSS, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 0.03 to 15 mM with a consequent increase in both $[Ca^{2+}]_i$ and force (Figure 6a). Summarized data obtained from five muscles are shown in Figure 6b. The results of computer fitting the curve to the logistic equation (2) are summarized in Table 2. The maximum effect shown in Table 2 is the calculated value. The pD_2 value for $[Ca^{2+}]_o$ to increase $[Ca^{2+}]_i$ (3.28 ± 0.13) is the apparent K_d value of $[Ca^{2+}]_o$ for binding at voltage-dependent Ca^{2+} channels opened by depolarization. The difference between the pD_2 value of $[Ca^{2+}]_o$ to increase $[Ca^{2+}]_i$ and that to produce contracture are a consequence of the nonlinear $[Ca^{2+}]_i$ -force relationship (Figure 7). When KRN2391 (10^{-4} M) was applied to muscle perfused with Ca^{2+} -free 90 mM KCl-PSS, $[Ca^{2+}]_i$ was not changed (Figure 6, Table 2). Although the increases in $[Ca^{2+}]_o$ produced the same increments in $[Ca^{2+}]_i$ in the absence (control) and the presence of KRN2391, the force produced by the increased $[Ca^{2+}]_o$ was smaller in the presence of KRN2391. Furthermore, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly decreased in the presence of KRN2391 ($P < 0.05$). These inhibitory effects of KRN2391 were reversible (Table 2). Figure 7 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and the presence of 10^{-4} M KRN2391 in 90 mM KCl-depolarized canine coronary artery. The curve in the presence of KRN2391 was different from that of the control and was shifted to the right. These results obtained from the analysis of $[Ca^{2+}]_i$ -force relation-

ship in the presence of KRN2391 suggest that KRN2391 reduces the Ca^{2+} -sensitivity of the contractile proteins.

Modification by methylene blue of the effect of KRN2391

Although methylene blue (10^{-5} M) reduced F_{340} and F_{380} slightly, the values of F_{340}/F_{380} and $ratio_{rc}$ were not changed. Thus, we have assumed that it is possible to estimate changes in $[Ca^{2+}]_i$ from the $ratio_{rc}$ even in the presence of methylene blue. Methylene blue (10^{-5} M) antagonized the inhibitory effect of KRN2391 on contracture produced by exposure to 30 mM KCl-PSS, but had no effect on KRN2391-induced reduction of $[Ca^{2+}]_i$ (Figure 8a). The concentration-effect curve for force was shifted to the right by methylene blue and the results of analysis of the concentration-effect curves are shown in Table 1. Although the pD_2 value and the maximum effect of KRN2391 in reducing $[Ca^{2+}]_i$ in the presence of methylene blue were the same as in its absence (control), the pD_2 value of KRN2391 for inhibition of contracture was slightly smaller in the presence of methylene blue. The slope parameter of the concentration-effect curve of KRN2391 for reduction of force of contraction was reduced by methylene blue. In 90 mM KCl-PSS, methylene blue antagonized the ability of KRN2391 to inhibit the force of contraction (Figure 8b). The rightward shift of the curve by methylene blue was greater in 90 mM KCl-PSS than in 30 mM KCl-PSS (Figure 8a,

Figure 5 (A) Relationship between $[Ca^{2+}]_i$ and force in the absence (control; $\circ$) and presence of KRN2391 ($\bullet$) or KRN2391 + glibenclamide 10^{-5} M ($\blacksquare$) in 30 mM KCl-PSS. (B) Relationship between $[Ca^{2+}]_i$ and force in the absence (control; $\circ$) and the presence of Compound 3 ($\bullet$) or Compound 2 ($\blacktriangle$) in 30 mM KCl-PSS. Control curves were obtained by decreasing $[Ca^{2+}]_o$ (a: 2.5, b: 1.0, c: 0.3, d: 0.1 mM $[Ca^{2+}]_o$) in 30 mM KCl. Each point of KRN2391, Compound 3 or Compound 2 (1: 3×10^{-8} , 2: 10^{-7} , 3: 3×10^{-7} , 4: 10^{-6} , 5: 3×10^{-6} , 6: 10^{-5} , 7: 3×10^{-5} , 8: 10^{-4} , 9: 3×10^{-4} , 10: 10^{-3} M) was adapted from Figures 3 and 4, respectively. Data are expressed as mean of 5–6 experiments. Vertical and horizontal bars are s.e.mean.

b). The increased $[Ca^{2+}]_i$ was not changed by KRN2391 in the presence of methylene blue (Figure 8b).

Effect of KRN2391 on U46619-induced contractures and increase in $[Ca^{2+}]_i$

When coronary arterial rings were perfused with U46619 (3×10^{-7} M), $[Ca^{2+}]_i$ increased transiently and then remained at an elevated level. The force of contraction was increased in a similar manner (Figure 9a). KRN2391 (10^{-8} – 10^{-4} M) reduced the increased $[Ca^{2+}]_i$, and the $[Ca^{2+}]_i$ was decreased below the basal level by KRN2391 (10^{-6} – 10^{-4} M). Thus, the maximum effect of KRN2391 was $145.7 \pm 8.0\%$ (Table 1). KRN2391 almost completely inhibited the force of contraction produced by U46619. The pD_2 values and the slope parameter for reduction of $[Ca^{2+}]_i$ and force of contraction were almost the same (Table 1).

Discussion

In this study, KRN2391 partially reduced $[Ca^{2+}]_i$ in a concentration-dependent manner in both 5 and 30 mM KCl-PSS, but failed to modify $[Ca^{2+}]_i$ in 90 mM KCl-PSS. The reduction of $[Ca^{2+}]_i$ caused by KRN2391 in 30 mM KCl-PSS

was antagonized by 10^{-5} M glibenclamide. The other K channel openers, cromakalim, pinacidil and nicorandil, each reduced the increase in $[Ca^{2+}]_i$ induced by 30 mM KCl, but that induced by 90 mM KCl was unaffected (Yanagisawa *et al.*, 1990). The KRN2391 reduction of $[Ca^{2+}]_i$ was thus influenced by the KCl concentration; a major characteristic of K channel openers, but not Ca^{2+} channel blockers (Yanagisawa *et al.*, 1990). In normal (5 mM KCl) PSS, KRN2391 reduced $[Ca^{2+}]_i$ below the basal level; a similar effect had also been observed with cromakalim, pinacidil and nicorandil (Yanagisawa *et al.*, 1990). Yanagisawa *et al.* (1990) suggested that certain Ca^{2+} channels are open in normal PSS, and that a hyperpolarization of the plasma membrane by a K channel opener is capable of closing this Ca^{2+} -entry pathway. Nelson *et al.* (1990) have shown that voltage-dependent Ca channels in arterial smooth muscle in the steady state can be open and very sensitive to membrane potential changes in a range that occurs in resistance arteries with tone, and that many vasodilators work, in part, through membrane hyperpolarization caused by ATP-sensitive K (K_{ATP}) channel activation. Although there have not been any reports about the effects of KRN2391 on the membrane potentials or membrane currents of vascular smooth muscle cells, KRN2391 increased ^{86}Rb efflux from rat aorta, indicating that it opened K channels (Kashiwabara *et al.*, 1991). This has also been observed with various other K channel openers (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast & Cook, 1989). Recently, sulphonylureas like glibenclamide have been shown to block cromakalim-induced electrical and mechanical changes in cardiac (Escande *et al.*, 1988; Fosset *et al.*, 1988; Sanguinetti *et al.*, 1988; Satoh *et al.*, 1990) and in vascular smooth muscle (Buckingham *et al.*, 1989; Eltze, 1989; Quast & Cook, 1989; Winquist *et al.*, 1989), suggesting a role for K_{ATP} channels (Standen *et al.*, 1989). The antagonism of KRN2391-induced reduction of $[Ca^{2+}]_i$ by glibenclamide indicates that, like other K channel openers, KRN2391 reduces $[Ca^{2+}]_i$ by hyperpolarizing the membrane of coronary arterial smooth muscle cells and thus closes Ca channels (Yanagisawa *et al.*, 1990). Therefore, it is conceivable that KRN2391 is one of the K_{ATP} channel openers, although the selectivity of glibenclamide for the K_{ATP} channel has not been established in smooth muscles.

Although in 30 mM KCl-PSS, KRN2391 reduced the force of contraction with a reduction of $[Ca^{2+}]_i$, the reduction of $[Ca^{2+}]_i$ is not the sole mechanism of its vascular relaxation. In 30 mM KCl-PSS, KRN2391 reduced $[Ca^{2+}]_i$ by about 40% but it almost abolished the force of contraction at high concentrations. In the presence of glibenclamide, the reduction of $[Ca^{2+}]_i$ was effectively inhibited, but the reduction of force of contraction remained. KRN2391 relaxed the artery without reducing the increased $[Ca^{2+}]_i$ in 90 mM KCl-PSS, and changed the relationship between $[Ca^{2+}]_i$ and force in 30 and 90 mM KCl-PSS. Therefore, in addition to its K channel opening action, KRN2391 reduces the sensitivity of contractile proteins to Ca^{2+} . This effect of KRN2391 was inhibited by methylene blue (Figure 8), a supposed guanylate cyclase inhibitor (Ignarro *et al.*, 1981). KRN2391 contains a nitroxy moiety within its structure and increased intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in canine coronary artery (in our laboratory, data not shown). Furthermore, the inhibitory effect of KRN2391 on contracture induced by noradrenaline was reduced by methylene blue or oxyhaemoglobin in the rat aorta (Kashiwabara *et al.*, 1991). Comp. 3, a denitrate derivative of KRN2391, did not reduce the sensitivity of contractile proteins to Ca^{2+} (Figure 5). These findings indicate that KRN2391 can also act as a nitrate in that it reduces the sensitivity of contractile proteins to Ca^{2+} . Nitroglycerin, a classical nitrate, also relaxes canine coronary arteries by this mechanism of action (Yanagisawa *et al.*, 1989), and sodium nitroprusside relaxes KCl-induced contractures accompanied by a small decrease in both $[Ca^{2+}]_i$ and ^{45}Ca uptake in the rat aorta (Karaki *et al.*, 1988). The amount of myosin light chain phosphorylation was reduced by sodium

Figure 6 (a) Changes in $[Ca^{2+}]_i$ and force produced by increasing $[Ca^{2+}]_o$ in a canine coronary arterial muscle depolarized with 90 mM KCl-PSS and the effects of KRN2391 on these changes. (b) Summarized data of control (O), 10^{-4} M KRN2391 (●) and washout (30 min, □) obtained from five experiments. Data are expressed as mean with s.e. mean shown by vertical bars. The force of contraction and the difference in the ratio, r_c , in the presence of 15 mM $CaCl_2$ and 90 mM KCl-PSS at 5 min were 9.7 ± 1.6 mN and 0.28 ± 0.06 , respectively, in the control group.

nitroprusside and 8-bromo cyclic GMP (Rapoport *et al.*, 1982; Draznin *et al.*, 1986). Thus, it is likely that nitrates, including KRN2391, relax canine coronary arteries by reducing the amount of myosin light chain phosphorylation normally associated with an increased $[Ca^{2+}]_i$ following Ca^{2+} influx on depolarization.

KRN2391 thus possesses a dual action, both as a K channel opener and a nitrate, and in this respect its action is similar to that of nicorandil in canine coronary arteries (Yanagisawa *et al.*, 1990). Since the effects of Comp. 2 and Comp. 3 (Figure 1) were blocked by 10^{-5} M glibenclamide and the relationships between $[Ca^{2+}]_i$ and force in the presence of Comp. 2 and Comp. 3 were the same as those in their absence, the mechanism of action of these two denitrate derivatives could be solely attributed to an opening of K channels. The order of

potency of this ability to reduce $[Ca^{2+}]_i$, which reflects a K channel opening action, was KRN2391 > Comp. 3 > Comp. 2. Comp. 2, in which a nitroxy moiety of KRN2391 is replaced by a hydroxy group, is much less potent than KRN2391. Comp. 3, with an acetoxy group, was about 10 times less potent than KRN2391 (Figure 3 and Table 1), but its maximum effect was slightly greater than that of KRN2391. Thus, the structure-activity relationships of pyridine derivatives as K channel openers indicates that the nitroxy substituent is very important for this activity. Such a structure-activity relationship has already been shown for nicorandil (Maruyama *et al.*, 1982; for review Edwards & Weston, 1990).

Finally, we also examined the effect of KRN2391 on the increased $[Ca^{2+}]_i$ and contracture induced by U46619, a

Table 2 Effects of KRN2391 on the $[Ca^{2+}]_i$ - $[Ca^{2+}]_o$ and force- $[Ca^{2+}]_o$ relationships in 90 mM KCl-depolarized canine coronary artery

	Maximum effect (%)	Increase in $[Ca^{2+}]_i$ pD_2	p	Maximum effect (%)	Increase in force pD_2	p
Control	158.7 ± 15.15	3.28 ± 0.13	0.90 ± 0.09	209.0 ± 35.98	2.65 ± 0.16	1.22 ± 0.14
KRN2391 (10^{-4} M)	170.8 ± 28.87	3.28 ± 0.10	1.05 ± 0.07	$114.5 \pm 22.79^*$	$2.23 \pm 0.07^*$	1.10 ± 0.07
Washout	170.0 ± 28.57	3.16 ± 0.21	0.94 ± 0.16	161.7 ± 31.46	2.45 ± 0.05	1.38 ± 0.11

Increase in $[Ca^{2+}]_i$ was calculated from the difference between the value obtained in Ca^{2+} -free 90 mM KCl and the value in the presence of $[Ca^{2+}]_o$. The maximum value of $[Ca^{2+}]_i$ in each experiment was usually obtained at 15 mM $[Ca^{2+}]_o$. Maximum effect (%) in $[Ca^{2+}]_i$ and force is the calculated values of each experiment by computer fitting to the logistic equation (2). p is the slope parameter. Significant difference from the control values: $^*P < 0.05$ ($n = 5$).

Figure 7 Relationship between $[Ca^{2+}]_i$ and force in the absence (control; $\circ$) and the presence of 10^{-4} M KRN2391 ($\bullet$). Each point was obtained from the data shown in Figure 6. Vertical and horizontal bars are s.e.mean ($n = 5$). The curves were fitted by eye.

stable thromboxane A_2 analogue. The contracture induced by U46619 was inhibited by KRN2391 in parallel with the reduction of $[Ca^{2+}]_i$ (Figure 8, Table 1). The $[Ca^{2+}]_i$ in the presence of 10^{-6} – 10^{-4} M KRN2391 was decreased below the basal level as in the absence of U46619. Since the pD_2 value of KRN2391 for reduction of $[Ca^{2+}]_i$ in the presence of U46619 was almost the same as that in 5 mM KCl-PSS, the effect of KRN2391 seems to be mainly due to K channel opening action. Thus, the tonic increase in $[Ca^{2+}]_i$ produced by U46619 is mainly due to Ca^{2+} influx through voltage-dependent Ca channels (Nelson *et al.*, 1990) as is the case with histamine in the coronary artery of the pig (Mori *et al.*, 1990).

In conclusion, KRN2391 acts as an N-K hybrid compound and the nitroxy moiety in KRN2391 is important not only for its action as a nitrate but also for its potency as a K channel opener.

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Figure 8 Influence of methylene blue on the ability of KRN2391 to reduce the increased $[Ca^{2+}]_i$ and force of contraction in high KCl-PSS. The effects of KRN2391 on $[Ca^{2+}]_i$ or the force produced by 30 mM KCl-PSS (a) and on the force produced by 90 mM KCl-PSS (b) in the absence ($\circ$) and the presence of 10^{-5} M methylene blue ($\bullet$). Methylene blue was added 20 min before exposure to high KCl-PSS and then KRN2391 was administered 30 min later. The concentrations of KRN2391 were increased cumulatively. The $[Ca^{2+}]_i$ and force are each expressed as a percentage of each value before the addition of KRN2391. Data are expressed as mean ($n = 5-6$); s.e.mean shown by vertical bars.

Figure 9 Effects of KRN2391 (10^{-8} – 10^{-4} M) on the increase in $[Ca^{2+}]_i$ and the force of contraction produced by 3×10^{-7} M U46619. (a) Typical traces of the force and $[Ca^{2+}]_i$ (indicated as fluorescence ratio). KRN2391 (10^{-8} – 10^{-4} M) was applied cumulatively. (b) The summarized data were obtained from five preparations. The increase in $[Ca^{2+}]_i$ (●) and the force of contraction (○) are expressed as a percentage of each effect to 90 mM KCl-PSS at 10 min. Data are expressed as mean with s.e.mean shown by vertical bars. Dotted lines indicate basal values.

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