

Cytoplasmic calcium and the relaxation of canine coronary arterial smooth muscle produced by cromakalim, pinacidil and nicorandil

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1 In order to investigate the vasodilator mechanisms of the K⁺ channel openers, cromakalim, pinacidil and nicorandil, we measured changes in cytoplasmic Ca²⁺ concentration ([Ca²⁺]_i) simultaneously with force by a microfluorimetric method using fura-2, a calcium indicator, in canine coronary arterial smooth muscle cells.

2 The three K⁺ channel openers all produced a concentration-dependent reduction of [Ca²⁺]_i in 5 and 30 mM KCl physiological salt solution (PSS) but failed to affect [Ca²⁺]_i in 45 and 90 mM KCl-PSS.

3 Cromakalim only partly inhibited (–45%) the 30 mM KCl-induced contractures, whereas pinacidil and nicorandil nearly abolished contractions produced by 45 mM, 90 mM and 30 mM KCl-PSS.

4 Tetrabutylammonium (TBA), a nonselective K⁺ channel blocker, or glibenclamide, a supposed adenosine 5'-triphosphate (ATP)-sensitive K⁺ channel blocker, abolished the reduction of [Ca²⁺]_i caused by the three K⁺ channel openers and the relaxant effect of cromakalim, whereas they only slightly attenuated the relaxant effects of pinacidil and nicorandil.

5 The increase in [Ca²⁺]_i produced by 45 or 90 mM KCl-PSS in the presence of pinacidil or nicorandil was abolished by 10^{–5} M verapamil, indicating that the increase in [Ca²⁺]_i was caused by the influx of extracellular Ca²⁺ and that pinacidil and nicorandil did not affect the voltage-dependent Ca²⁺ channel directly.

6 The [Ca²⁺]_i-force relationship in the presence of cromakalim was not distinguishable from that of control.

7 The [Ca²⁺]_i-force curve was shifted to the right by pinacidil and nicorandil.

8 These results show that cromakalim is a more specific K⁺ channel opener than pinacidil and nicorandil, and that vasodilatation produced by cromakalim in this study is predominantly a result of a reduction of [Ca²⁺]_i due to the closure of voltage-dependent Ca²⁺ channels by hyperpolarization. In contrast, additional mechanisms are involved in the vasodilator actions of pinacidil and nicorandil. One of these is related to a reduction in the sensitivity of contractile proteins to Ca²⁺. The latter mechanism of nicorandil is akin to that of nitroglycerin. K⁺ channels opened by these K⁺ channel openers may be ATP-sensitive ones which are blocked by glibenclamide.

Introduction

Increasing attention has recently been paid to vasodilator drugs which activate or open K⁺ channels in vascular smooth muscle and which eventually increase coronary blood flow or lower blood pressure. These drugs have been called 'K⁺ channel activators or openers' and include nicorandil (Taira *et al.*, 1979; Yanagisawa *et al.*, 1979), pinacidil (Arrigoni-Martelli *et al.*, 1980; Bray *et al.*, 1987) and cromakalim (BRL 34915; Hamilton *et al.*, 1986; Weir & Weston, 1986a,b), although nicorandil also has a nitrate-like action (Taira, 1987; 1989). In canine atrial muscle, these three K⁺ channel openers reduce the force of contraction exclusively by opening K⁺ channels, because their negative inotropic effects are totally blocked by the quaternary ammonium K⁺ channel blockers, tetraethylammonium (TEA) or tetrabutylammonium (TBA) (Yanagisawa *et al.*, 1988; 1989a).

In vascular smooth muscle cells the resting membrane potential is less negative than the K⁺ equilibrium potential (Furukawa *et al.*, 1981). When the membrane is hyperpolarized towards the K⁺ equilibrium potential by K⁺ channel openers, excitation-induced Ca²⁺ influx via voltage-dependent Ca²⁺ channels is thought to decrease (Cook, 1988; Weston, 1989). However, no direct measurement of the effects of K⁺ channel openers on intracellular Ca²⁺ concentration ([Ca²⁺]_i) has yet been obtained. Furthermore, as K⁺ channel

openers are structurally different, it is possible that some possess other vasodilator mechanisms.

Although [Ca²⁺]_i is one of the main determinants of mechanical activity of vascular smooth muscle, various studies have shown that it is not the only determinant of this activity (Kamm & Stull, 1989; Karaki, 1989). By improving the methods of Ozaki *et al.* (1987) and Bruschi *et al.* (1988) we have recently developed a microfluorimetric method, using the Ca²⁺ indicator, fura-2 (Gryniewicz *et al.*, 1985), to measure simultaneously [Ca²⁺]_i with force in isolated vascular rings (Yanagisawa *et al.*, 1989b). In the present study we used this method to investigate the effects of K⁺ channel openers on [Ca²⁺]_i and tension of canine isolated coronary arterial rings. We further studied how their effects were modified by the nonselective K⁺ channel blocker, TBA (Stanfield, 1983) and the supposed adenosine 5'-triphosphate (ATP)-sensitive K⁺ channel blocker, glibenclamide (Cook, 1988; Castle *et al.*, 1989), to identify what kind of K⁺ channels are involved.

Methods

Tissue preparation

Hearts were obtained from mongrel dogs of either sex, weighing 5 to 13 kg, anaesthetized with pentobarbitone sodium (30 mg kg^{–1}, i.v.). Coronary arterial rings (0.5–1.5 mm in diameter, about 1 mm in width) were dissected and connective

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tissues were carefully removed in a dissecting chamber under a binocular microscope. The endothelium was removed by gentle rubbing and the luminal side was turned to face outwards.

Fluorescence measurements

Coronary arterial rings were exposed to 10 μM fura-2 acetoxy-methyl 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 fura-2 AM in PSS (Poenie *et al.*, 1986). After the fura-2 loading, muscles were rinsed with normal PSS for more than 1 h and used for experiments. Fluorescence was measured in a fluorimeter equipped with a dual wavelength excitation device (CAM-200, Japan Spectroscopic, Tokyo, Japan) connected to an inverted microscope (TMD, Nikon, Tokyo, Japan). For further details see Yanagisawa *et al.* (1989b). The fluorescence image was obtained by focusing on the smooth muscle cells in the medial layer with a Nikon CF UV (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). The photosignals and the mechanical activity were measured simultaneously and both recorded on a chart recorder (Recti-horiz-8k, NEC-Sanei, Tokyo, Japan). They were also digitized by A/D converters and fed into a personal computer for further calculation and graphical analysis (Himpens & Somlyo, 1988) by a microcomputer (PC-9801, NEC, Tokyo, Japan).

Every experiment commenced with perfusion of 90 mM KCl-PSS for 10 min. We subtracted the F_{340} and F_{380} values due to autofluorescence from the corresponding values of F_{340} and F_{380} obtained under conditions of fura-2 loading to derive a recalculated ratio_{re}. Changes in the ratio_{re} (changes in $[\text{Ca}^{2+}]_i$) 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 and MnCl_2 , affected fluorescence signals at the concentrations used.

Drugs and solutions

The composition (mmol l⁻¹) of normal or 5 mM-KCl physiological salt solution (PSS) was as follows: NaCl 140, KCl 5, CaCl_2 2.5, MgCl_2 2.5, glucose 11.1, HEPES 3 (pH 7.4). The solution was equilibrated with 100% O_2 at 37°C. High KCl-PSS was made by substituting NaCl with equimolar KCl. Ca^{2+} -free PSS was made by removing CaCl_2 from normal PSS and sometimes by adding 1 mM EGTA to the solution.

Drugs and chemicals were obtained from the following sources: cromakalim (BRL 34915; Beecham, Harlow, U.K.), nicorandil (Chugai, Tokyo, Japan), pinacidil monohydrate (Shionogi, Osaka, Japan), verapamil hydrochloride (Eisai, Tokyo, Japan), glibenclamide (Hoechst Japan, Tokyo, Japan and Yamanouchi, Tokyo, Japan), EGTA (glycol-etherdiamine-tetraacetic acid), fura-2 AM, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and pluronic F-127 (Dojin, Kumamoto, Japan), TBA (tetrabutylammonium chloride), DMSO (dimethyl sulphoxide) and Triton X-100 (Wako, Tokyo, Japan). 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 mmol l⁻¹. Cromakalim was dissolved in 70% ethanol to give a concentration of 10 mmol l⁻¹. Pinacidil was dissolved in 0.1 N HCl to give a concentration of 200 mmol l⁻¹. Other drugs were dissolved in distilled water to the desired concentrations. From these stock solutions the

appropriate concentrations were obtained by diluting with PSS.

Evaluation of responses

The concentration-effect curves for K^+ channel openers in reducing the force of contraction and increase in $[\text{Ca}^{2+}]_i$ produced by high KCl-PSS were expressed as a % reduction from the pre-drug values of force of contracture or increase in $[\text{Ca}^{2+}]_i$, and computer fitted to a logistic equation:

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

where E is the normalized response, M is the maximum response, A is the drug concentration, K is the EC_{50} value of each drug and p is the slope parameter (Parker & Waud, 1971). When the maximum response of each drug was not obtainable because of its limited solubility, M and p were fixed at 100 and 1, respectively. The concentration-effect curves for K^+ channel openers in reducing $[\text{Ca}^{2+}]_i$ below the basal level in 5 mM KCl-PSS were also computer fitted to equation (1). The maximum response, M, however, was the maximum reduction of $[\text{Ca}^{2+}]_i$ produced on perfusion with Ca^{2+} -free-PSS containing 1 mM EGTA which was taken as 100%.

The concentration-effect curves for extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) were also computer fitted to a logistic equation:

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

where E is the response normalized by the differences between basal values and those of 90 mM KCl at 10 min, A is $[\text{Ca}^{2+}]_o$, K is EC_{50} value of $[\text{Ca}^{2+}]_o$, and p is slope parameter. EC_{50} values were presented as pD_2 ($\text{pD}_2 = -\log \text{EC}_{50}$).

Experimental values are given as means or means \pm s.e.mean. Statistical significance of differences between mean values was estimated by Student's *t* test. A *t* test for paired comparison was used when it was applicable. A *P* value smaller than 0.05 was considered to be significant.

Results

Effect of cromakalim on $[\text{Ca}^{2+}]_i$ and KCl-induced contractures

In canine coronary artery loaded with fura-2, perfusion with 30 mM KCl-PSS increased $[\text{Ca}^{2+}]_i$ and elicited a contracture. Cromakalim (10^{-7} – 3×10^{-5} M) reduced both $[\text{Ca}^{2+}]_i$ and contracture in a concentration-dependent manner (Figure 1a). These effects of cromakalim could be reversed by washing. The summarized data from 7 muscles are shown in Figure 1b and the results of the analysis of the concentration-effect curves for cromakalim to reduce the KCl-induced contracture and increase in $[\text{Ca}^{2+}]_i$ are summarized in Table 1. The pD_2 ($-\log \text{EC}_{50}$) values of cromakalim required to reduce the increase in $[\text{Ca}^{2+}]_i$ were not significantly higher than those needed to inhibit contracture.

The effects of cromakalim on KCl-induced changes were dependent on the concentration of KCl. When the arterial rings were perfused with 45 mM KCl-PSS, cromakalim had no effect on either the force of contracture or the increase in $[\text{Ca}^{2+}]_i$ (Figure 2a). In 5 mM KCl-PSS, cromakalim reduced $[\text{Ca}^{2+}]_i$ from the basal level, whereas the resting tone was not affected. When extracellular Ca^{2+} was removed by perfusion with Ca^{2+} -free-PSS containing 1 mM EGTA, $[\text{Ca}^{2+}]_i$ was reduced further (Figure 2b). There was no difference between the respective pD_2 values for cromakalim-induced changes in both $[\text{Ca}^{2+}]_i$ and force observed in the presence of 5 mM and 30 mM KCl, although the membrane potentials under these conditions should have been different.

Modification by glibenclamide and TBA of the effects of cromakalim

Figure 3a shows the modification by glibenclamide of the effects of 10^{-5} M cromakalim on the force of contracture and

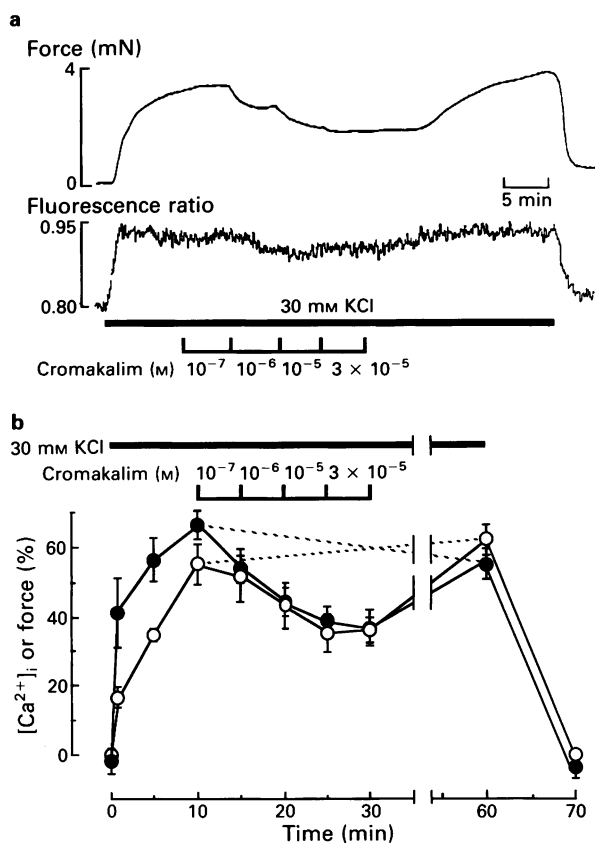


Figure 1 (a) Effects of cromakalim (10⁻⁷–3 × 10⁻⁵ M) on the force of contracture and increase in [Ca²⁺]_i (measured as fluorescence ratio) produced by 30 mM KCl-PSS in a single experiment in canine coronary artery. (b) The summarized data obtained from 7 arterial rings. The force of contracture (○) and the increase in [Ca²⁺]_i (●) are expressed as a percentage of the 10 min responses to 90 mM KCl-PSS in this and subsequent figures; vertical lines show s.e.mean.

the increase in [Ca²⁺]_i in canine coronary artery perfused with 30 mM KCl-PSS. Cromakalim (10⁻⁵ M) reduced both [Ca²⁺]_i and the force of contracture. When glibenclamide (10⁻⁶ and 10⁻⁵ M) was applied 10 min after perfusion with 30 mM KCl-PSS, no change in the increase in [Ca²⁺]_i or force was observed. In contrast the ability of cromakalim to reduce the increase in [Ca²⁺]_i and force were greatly attenuated by exposure to glibenclamide at 10⁻⁶ M and abolished at 10⁻⁵ M (Figure 3b). The effects of cromakalim were also blocked by 10⁻³ M TBA (Figure 4). Although TBA *per se* had little effect

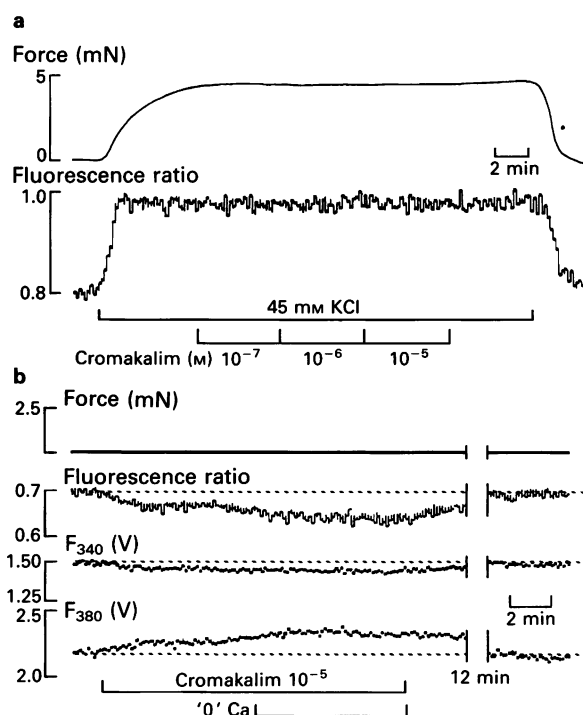


Figure 2 (a) Effects of cromakalim on force of contracture and the increase in [Ca²⁺]_i (measured as fluorescence ratio) of a canine coronary artery perfused with 45 mM KCl-PSS. (b) Effects of cromakalim on the resting force and [Ca²⁺]_i derived from fluorescence measurements in 5 mM KCl-PSS and Ca²⁺-free PSS containing 1 mM EGTA.

on the force of contracture and the increase in [Ca²⁺]_i in 30 mM KCl-PSS, its effects persisted after washout, since [Ca²⁺]_i after washout in 5 mM KCl-PSS was higher than the basal level (Figure 4a,b). In 5 mM KCl-PSS glibenclamide affected neither [Ca²⁺]_i nor the resting tension, whereas TBA slightly increased [Ca²⁺]_i by about 10% without changing the resting tension (data not shown).

Effects of pinacidil on KCl-induced contractures and increases in [Ca²⁺]_i

In 5 mM KCl-PSS, pinacidil reduced [Ca²⁺]_i below the basal level without changing the resting tension (Table 1). The reduction of [Ca²⁺]_i produced by 10⁻⁵ M pinacidil was abolished by 10⁻³ M TBA (n = 3). When the coronary arterial rings were perfused with 30 mM KCl-PSS, pinacidil, like cromakalim, reduced the increase in [Ca²⁺]_i with a pD₂ value of

Table 1 Effects of cromakalim, pinacidil and nicorandil on the force of contracture and [Ca²⁺]_i

KCl (mM)	(n)	Reduction of force			Reduction of [Ca ²⁺] _i		
		Maximum response (%)	pD ₂	p	Maximum response (%)	pD ₂	p
Cromakalim							
5	(7)		no effect (< 10 ⁻⁵ M)		60.1 ± 10.5	6.66 ± 0.17	1.07 ± 0.04
30	(7)	45.1 ± 6.4	6.04 ± 0.20	1.11 ± 0.13	40.1 ± 5.5	6.55 ± 0.11	0.94 ± 0.18
45	(6)		no effect (< 10 ⁻⁵ M)			no effect (< 10 ⁻⁵ M)	
Pinacidil							
5	(7)		no effect (< 3 × 10 ⁻⁴ M)		79.5 ± 8.6	5.40 ± 0.11	1.05 ± 0.10
30	(6)	100 ^a	5.11 ± 0.08	0.79 ± 0.06	44.8 ± 6.7	4.94 ± 0.16	1 ^a
45	(7)	100 ^a	4.47 ± 0.04	1.07 ± 0.04		no effect (< 3 × 10 ⁻⁴ M)	
90	(5)	100 ^a	4.16 ± 0.33	1.21 ± 0.26		no effect (< 10 ⁻⁴ M)	
Nicorandil							
30	(7)	100 ^a	4.33 ± 0.18	1.04 ± 0.17	35.9 ± 4.0	4.57 ± 0.9	1 ^a
45	(6)	100 ^a	3.72 ± 0.04	1.29 ± 0.19		no effect (< 10 ⁻³ M)	
90	(8)	100 ^a	3.64 ± 0.12	1.02 ± 0.13		no effect (< 10 ⁻³ M)	

The concentration-effect curves for cromakalim, pinacidil and nicorandil were analysed by computer fitting to a logistic equation: E = 100 - M × A^p / (A^p + K^p) where E is normalized response (basal value = 100%). M is maximum response, A is drug concentration. K is EC₅₀ value of each drug and p is slope parameter. EC₅₀ values were presented as pD₂ (pD₂ = -log EC₅₀). ^a M and p were fixed at 100 and 1, respectively.

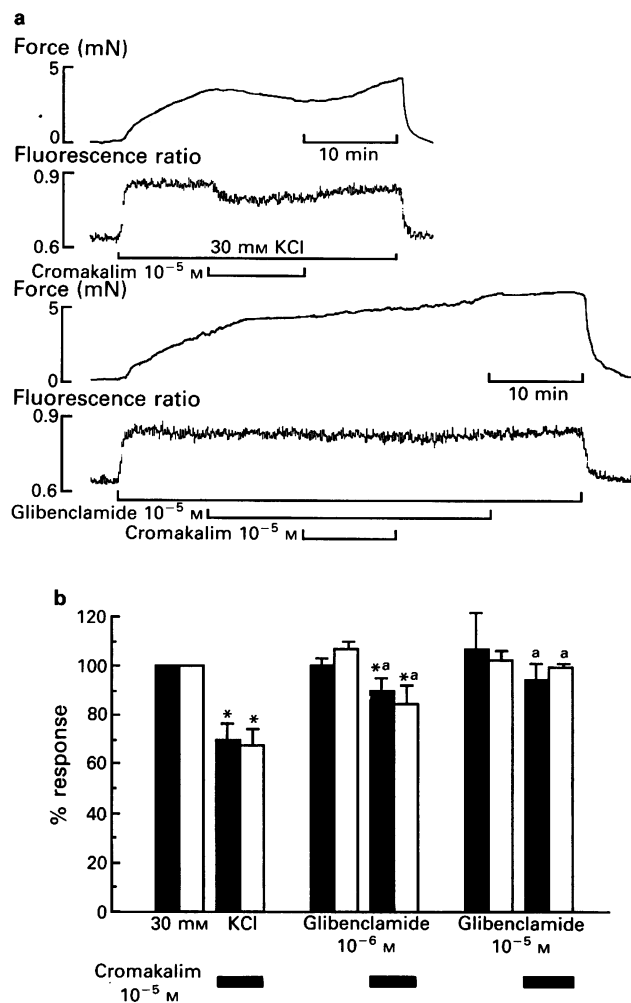


Figure 3 (a) Modification by glibenclamide (10^{-5} M) of the effects of cromakalim (10^{-5} M) on the force of contracture and the increase in $[Ca^{2+}]_i$ in a single experiment in canine coronary artery depolarized by 30 mM KCl-PSS. (b) Summarized data, expressed as a percentage of the values obtained just before the administration of cromakalim or glibenclamide ($n = 5-9$). * $P < 0.05$ compared with control values. * $P < 0.05$ compared with values in the presence of cromakalim 10^{-5} M alone. Open columns, force of contracture; solid columns, $[Ca^{2+}]_i$.

4.94 ± 0.16 . The 30 mM KCl-induced contracture was, however, nearly abolished by 10^{-4} M pinacidil with a pD_2 value of 5.11 ± 0.08 (Figure 5a; Table 1). Thus, although the pD_2 value of pinacidil for mechanical inhibition was slightly but not significantly greater than that for reduction of $[Ca^{2+}]_i$, the maximum effect on the former was much larger than that on the latter. In 45 mM KCl-PSS, pinacidil, in contrast to cromakalim, inhibited the force of contracture in a concentration-dependent manner, whereas the increase in $[Ca^{2+}]_i$ was not changed by pinacidil at all. Even during the washout of pinacidil the increase in $[Ca^{2+}]_i$ did not change (Figure 5b; Table 1). In 90 mM KCl-PSS, pinacidil inhibited the force of contracture without modifying the increase in $[Ca^{2+}]_i$. These results suggest that pinacidil, in addition to its K^+ channel opening action, is able to inhibit the high KCl-induced contracture without reducing the increase in $[Ca^{2+}]_i$. The results of the analysis of the concentration-effect curves for pinacidil are summarized in Table 1.

Effects of nicorandil on KCl-induced contractures and increases in $[Ca^{2+}]_i$

Without changing the resting tension in 5 mM KCl-PSS, 10^{-4} M nicorandil reduced $[Ca^{2+}]_i$ below the basal level, an effect which was blocked by 10^{-3} M TBA. When arterial rings were perfused with 30 mM KCl-PSS, nicorandil, like cromaka-

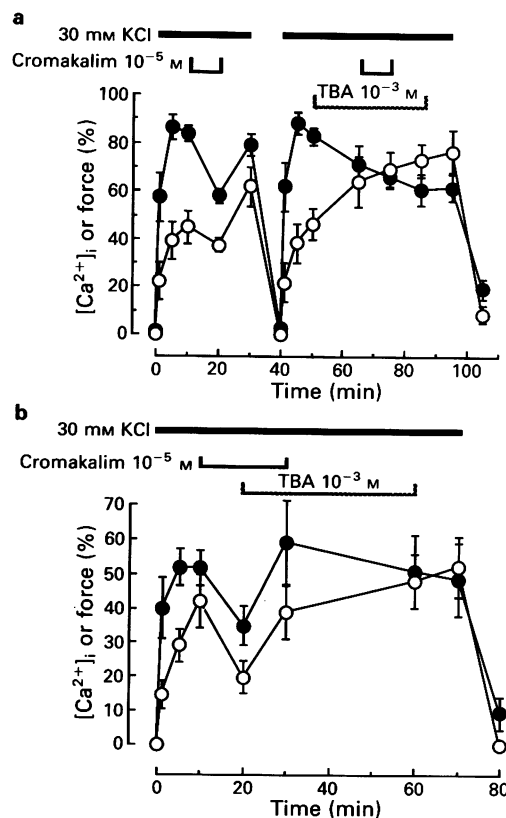


Figure 4 Modification by tetrabutylammonium (TBA, 10^{-3} M) of the effects of cromakalim (10^{-5} M) in canine coronary arteries perfused with 30 mM KCl-PSS. (a) Cromakalim was applied in the presence of TBA ($n = 5$). (b) TBA was applied after the effects of cromakalim had become steady ($n = 6$). (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

lim and pinacidil, reduced the increase in $[Ca^{2+}]_i$ with a pD_2 value of 4.57 ± 0.19 . The KCl-induced contracture was, however, nearly abolished by 10^{-3} M nicorandil. When the maximum effect was assumed to be 100%, its pD_2 value for inhibition of contracture was 4.33 ± 0.18 . Thus, although this pD_2 value for inhibition of contracture was slightly but not significantly smaller than that associated with a reduction of the increase in $[Ca^{2+}]_i$, the maximum effect was much larger on the former than on the latter (Table 1). In 45 mM KCl-PSS, nicorandil inhibited the force of contracture in a concentration-dependent manner, whereas the increase in $[Ca^{2+}]_i$ was not changed by nicorandil. Even during the washout of nicorandil, there was no change in the increase in $[Ca^{2+}]_i$, and in 90 mM KCl-PSS, nicorandil inhibited the force of contracture without changing $[Ca^{2+}]_i$ (Table 1). These results indicate that nicorandil also has an effect additional to that of K^+ channel opening in inhibiting the high KCl-induced contracture without reducing the increase in $[Ca^{2+}]_i$. The relaxant potency of nicorandil was dependent on KCl concentration; with lower pD_2 values the greater the presumed muscle depolarization.

Modification by TBA or glibenclamide of the effects of pinacidil and nicorandil

TBA (10^{-3} M) or glibenclamide (10^{-5} M) antagonized the ability of pinacidil (10^{-4} M and 3×10^{-4} M) to reduce $[Ca^{2+}]_i$ following exposure to 30 mM KCl-PSS, but had little effect on pinacidil-induced inhibition of contracture (Figure 6). Glibenclamide (10^{-5} M) also abolished the ability of nicorandil (10^{-4} and 10^{-3} M) to reduce $[Ca^{2+}]_i$, and only slightly attenuated its inhibition of contracture (Figure 7).

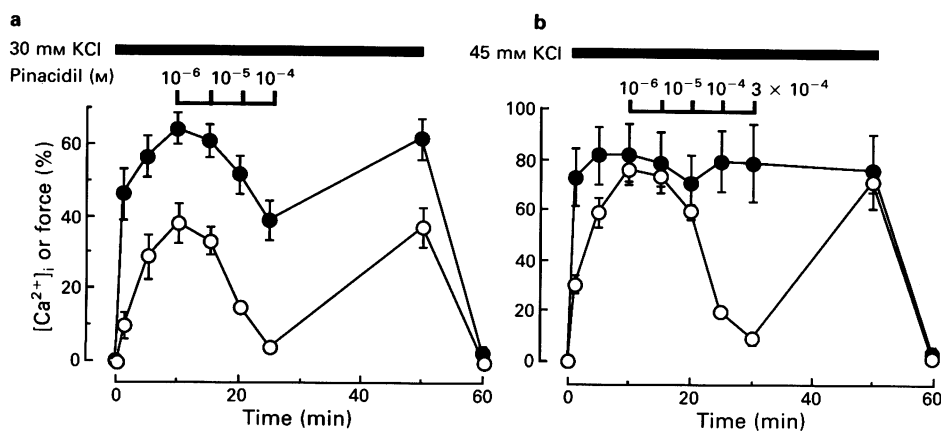


Figure 5 (a) The summarized data of the effects of pinacidil (10^{-6} – 3×10^{-4} M) on the force of contracture (○) and increase in $[Ca^{2+}]_i$ (●) in 6 canine coronary arteries depolarized by 30 mM KCl-PSS. (b) Summarized data obtained from 8 muscles perfused with 45 mM KCl.

Effect of verapamil on the increase in $[Ca^{2+}]_i$ induced by high KCl-PSS in the presence of pinacidil or nicorandil

The observation that pinacidil or nicorandil relaxed coronary artery contracted by 45 or 90 mM KCl-PSS without changing the increase in $[Ca^{2+}]_i$ prompted a series of experiments to determine whether the change in $[Ca^{2+}]_i$ was verapamil-sensitive. Figure 8 shows the results of such experiments for pinacidil or nicorandil in arterial rings exposed to 45 mM KCl-PSS. Without changing the increase in $[Ca^{2+}]_i$, pinacidil (10^{-4} M) reduced the force of contracture induced by 45 mM and 90 mM KCl-PSS to $22.4 \pm 6.3\%$ ($n = 7$) and $47.9 \pm 11.6\%$ ($n = 6$) (force produced by 45 mM or 90 mM KCl-PSS at 10 min = 100%), respectively. In the presence of 45 mM KCl-PSS containing 10^{-4} M pinacidil, addition of 10^{-5} M verapamil abolished the remaining force and the increase in $[Ca^{2+}]_i$ (Figure 8a,b). Nicorandil 10^{-3} M reduced the force of contracture induced by 45 mM or 90 mM KCl-PSS to $15.9 \pm 5.8\%$ ($n = 6$) and $24.9 \pm 2.2\%$ ($n = 8$), respectively, with no effect on the increase in $[Ca^{2+}]_i$. Exposure to 10^{-5} M verapamil in the presence of 10^{-3} M nicorandil abolished the remaining force and the increase in Ca^{2+} (Figure 8c,d).

Effect of pinacidil or nicorandil on extracellular Ca^{2+} -induced changes in mechanical activity and $[Ca^{2+}]_i$ in KCl-depolarized coronary artery

As previously described, the relaxant potency of both pinacidil and nicorandil was dependent on the KCl concentration (Table 1). To clarify whether the extent of depolarization or the increase in $[Ca^{2+}]_i$ has the major influence on the relaxant effects of pinacidil and nicorandil, coronary muscle was at

first perfused with Ca^{2+} -free PSS for 10 min and then with Ca -free 90 mM KCl-PSS. Removal of extracellular Ca^{2+} produced a reduction of $[Ca^{2+}]_i$ below the basal level ($-27 \pm 2\%$; expressed as a % of the increase following 10 min exposure to 90 mM KCl-PSS), whereas the force was not changed. After removal of extracellular Ca^{2+} by perfusion with Ca^{2+} -free PSS, exposure of the muscle to 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ ($-31 \pm 2\%$) or any change in force. During exposure to 90 mM 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 9, left panel). The results of computer fitting of the curve to the logistic equation (2) are summarized in Table 2. The maximum is the difference between values at Ca^{2+} -free and 15 mM $[Ca^{2+}]_o$. The pD_2 values for $[Ca^{2+}]_o$ of 3.05 ± 0.23 indicate the apparent K_D values of $[Ca^{2+}]_o$ for the binding constant at voltage-dependent Ca^{2+} channels presumed to be opened by depolarization. The relationship between $[Ca^{2+}]_o$ and force of contracture was also analysed and is summarized in Table 2. The maximum force produced by 90 mM KCl was $239 \pm 41\%$, the pD_2 value for $[Ca^{2+}]_o$ was 2.62 ± 0.14 , and the slope parameter was 1.22 ± 0.12 . The difference between pD_2 values for the influence of $[Ca^{2+}]_o$ on the increase in $[Ca^{2+}]_i$ and the increase in force are a consequence of the steepness of $[Ca^{2+}]_i$ -force relationship (Figure 10, control).

When pinacidil (10^{-4} M) or nicorandil (3×10^{-4} M) was applied to coronary arterial muscle perfused with Ca^{2+} -free 90 mM KCl-PSS, $[Ca^{2+}]_i$ was not changed. Although increases in $[Ca^{2+}]_o$ produced the same increases in $[Ca^{2+}]_i$ in the presence of pinacidil or nicorandil as in control, the force produced by the increased $[Ca^{2+}]_i$ was much smaller than that of control and the threshold values for $[Ca^{2+}]_o$ and

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

	Maximum response (%)	Increase in force		Increase in $[Ca^{2+}]_i$		
		pD_2	p	Maximum response (%)	pD_2	p
Control	239 ± 41	2.62 ± 0.14	1.22 ± 0.12	160 ± 23	3.05 ± 0.23	0.96 ± 0.14
Pinacidil 10^{-4} M	$82 \pm 29^*$	$2.05 \pm 0.15^*$	1.39 ± 0.17	146 ± 24	2.86 ± 0.18	0.93 ± 0.08
Washout	217 ± 41	2.71 ± 0.07	1.30 ± 0.09	150 ± 20	3.17 ± 0.28	0.96 ± 0.15
Control	309 ± 85	2.53 ± 0.06	1.39 ± 0.07	174 ± 25	2.99 ± 0.07	0.88 ± 0.11
Nicorandil 3×10^{-4} M	$138 \pm 24^*$	$1.97 \pm 0.13^*$	1.29 ± 0.07	163 ± 19	2.93 ± 0.14	0.90 ± 0.11
Washout	285 ± 63	2.59 ± 0.06	1.37 ± 0.23	160 ± 30	3.11 ± 0.10	0.93 ± 0.09

Increase in $[Ca^{2+}]_i$ was calculated from the difference between the value obtained in Ca^{2+} -free 90 mM KCl-PSS and the maximum value of $[Ca^{2+}]_i$ in each experiment (usually at 15 mM $[Ca^{2+}]_o$). * $P < 0.05$ compared with control values; p = slope parameter.

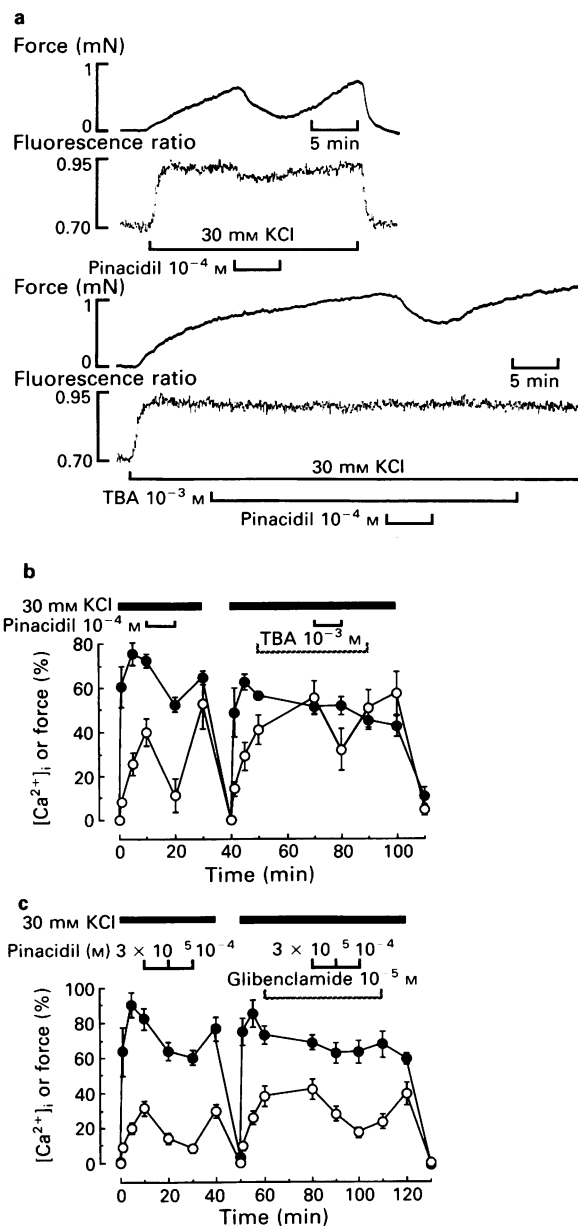


Figure 6 (a) Modification by tetrabutylammonium (TBA, 10^{-3} M) of the effects of pinacidil (10^{-4} M) in a single experiment in canine coronary artery perfused with 30 mM KCl-PSS. (b) Summarized data obtained from 4 muscles. (c) Summarized data of the influence of glibenclamide (10^{-5} M) on the effects of pinacidil (3×10^{-5} – 10^{-4} M) in 6 canine coronary arterial muscles perfused with 30 mM KCl-PSS. (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

$[Ca^{2+}]_i$ required to generate force were increased (Figure 9). Furthermore, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly reduced in the presence of pinacidil or nicorandil. The inhibitory effects of pinacidil or nicorandil were reversible (Figure 9 and Table 2).

Effects of pinacidil or nicorandil on the relationship between $[Ca^{2+}]_i$ and force in KCl-depolarized canine coronary artery

Figure 10 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and presence of pinacidil (10^{-4} M) or nicorandil (3×10^{-4} M) in depolarized canine coronary artery. The curve in the presence of pinacidil and nicorandil was different from that of the control and was shifted to the right and downward. These results obtained from the analysis of $[Ca^{2+}]_i$ -tension relationship in the presence of pinacidil or nicorandil suggest

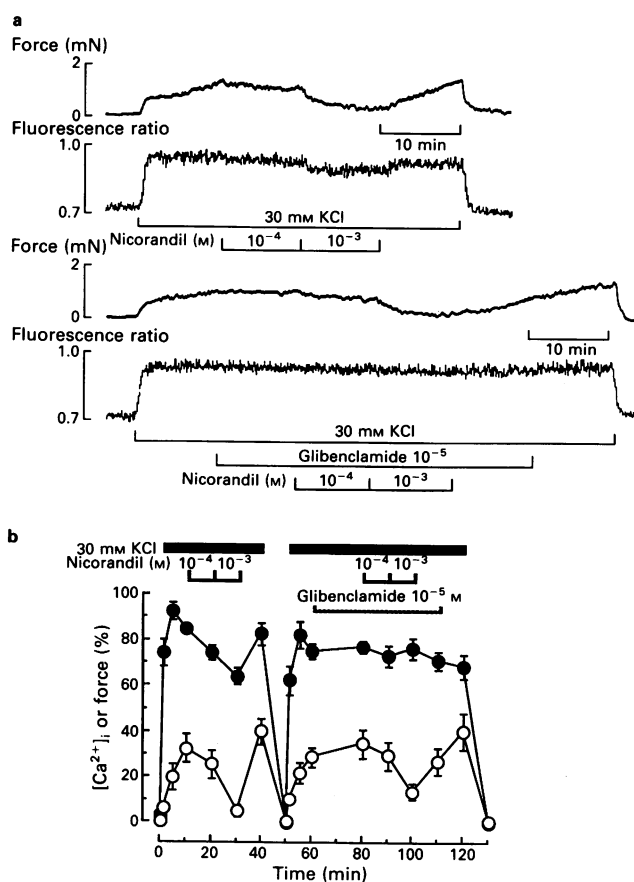


Figure 7 Modification by glibenclamide (10^{-5} M) of the effects of nicorandil (10^{-4} – 10^{-3} M) in a single experiment in canine coronary artery perfused with 30 mM KCl-PSS. (b) Summarized data obtained from 6 muscles. (●) Increase in $[Ca^{2+}]_i$, (○) force of contracture.

that both agents reduce the Ca^{2+} -sensitivity of the contractile proteins.

Discussion

In the present study, presumed depolarization of canine coronary artery by 30–90 mM KCl-PSS produced a concentration-dependent increase in $[Ca^{2+}]_i$ and force. A reduction of $[Ca^{2+}]_i$ below the basal level did not affect the tone while the threshold increase in $[Ca^{2+}]_i$ necessary to generate tension was about 10% of basal level. The observed increase in $[Ca^{2+}]_i$ and tension was verapamil-sensitive and thus was almost certainly associated with an increase in Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels (Godfraind *et al.*, 1986; Yanagisawa *et al.*, 1989).

When tissues were exposed to PSS containing KCl (≤ 30 mM), the three K^+ channel openers, cromakalim, pinacidil and nicorandil, each reduced the increase in $[Ca^{2+}]_i$, whereas when the KCl concentration was 45 mM or greater the increase in $[Ca^{2+}]_i$ was unaffected. Thus, the effects of K^+ channel openers in reducing the increase in $[Ca^{2+}]_i$ may only be achieved when the resultant membrane potential associated with the presence of a raised KCl concentration is less negative than the equilibrium potential for K^+ (Yanagisawa & Taira, 1980; Furukawa *et al.*, 1981), and when the K^+ channel openers can hyperpolarize the membrane potential to reduce the opening probability of voltage-dependent Ca^{2+} channels (Weston, 1987). In normal (5 mM KCl) PSS, cromakalim, pinacidil and nicorandil reduced $[Ca^{2+}]_i$ below the basal level and this effect of pinacidil and nicorandil was blocked by TBA. This suggests that certain Ca^{2+} channels are open in normal PSS and that a presumed hyperpolarization of the plasma

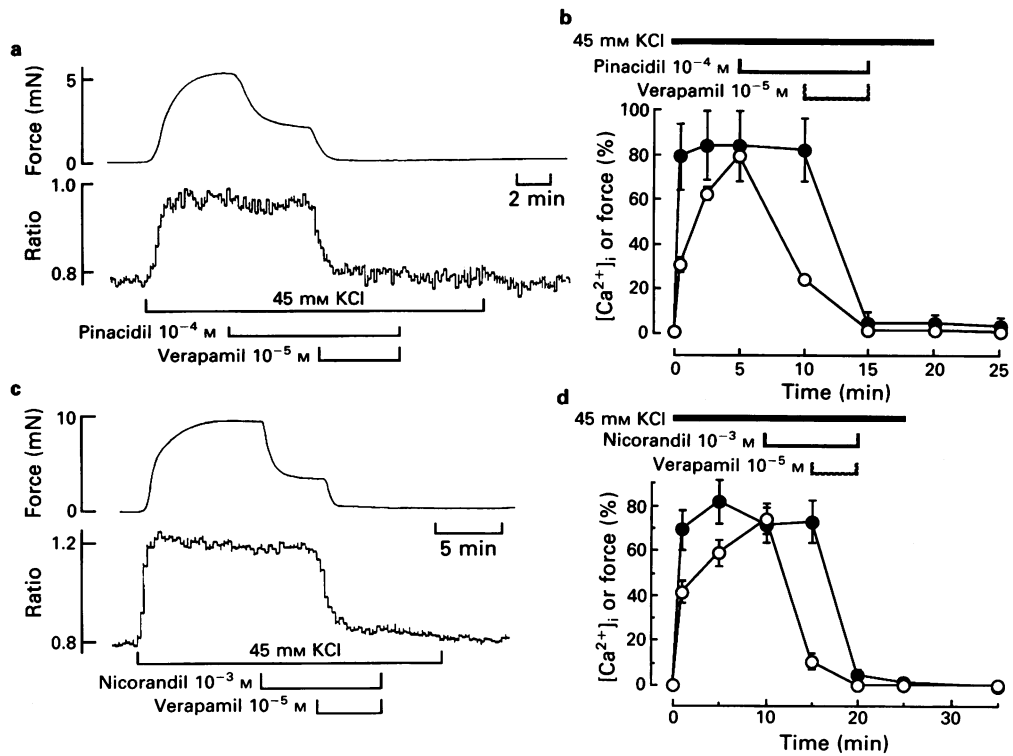


Figure 8 Effects of 10⁻⁴ M pinacidil (a,b) and 10⁻³ M nicorandil (c,d) and 10⁻⁵ M verapamil on changes in the increase in [Ca²⁺]_i (●) and force of contracture (○) produced by 45 mM KCl-PSS. Summarized data obtained from 7 muscles for pinacidil (b) and from 6 muscles for nicorandil (d) are also shown.

membrane by a K⁺ channel opener is capable of closing this Ca²⁺-entry pathway.

Although the vasodilator effect of K⁺ channel openers is blocked by several nonselective K⁺ channel blockers such as procaine, 4-aminopyridine and tetraethylammonium (TEA) (Wilson *et al.*, 1988), the identity of the K⁺ channels involved is uncertain. Recently, sulphonylureas like glibenclamide have been shown to block cromakalim-induced changes in cardiac

(Fosset *et al.*, 1988; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) and vascular smooth muscle (Buckingham *et al.*, 1989; Quast & Cook, 1989; Winquist *et al.*, 1989; Eltze, 1989), suggesting a role for ATP-sensitive K⁺ channels (Standen *et al.*, 1989). In the present study glibenclamide blocked the effects of the three K⁺ channel openers in reducing [Ca²⁺]_i, although rather high concentrations (≥ 10⁻⁶ M) were needed. The finding that glibenclamide affected neither [Ca²⁺]_i nor force

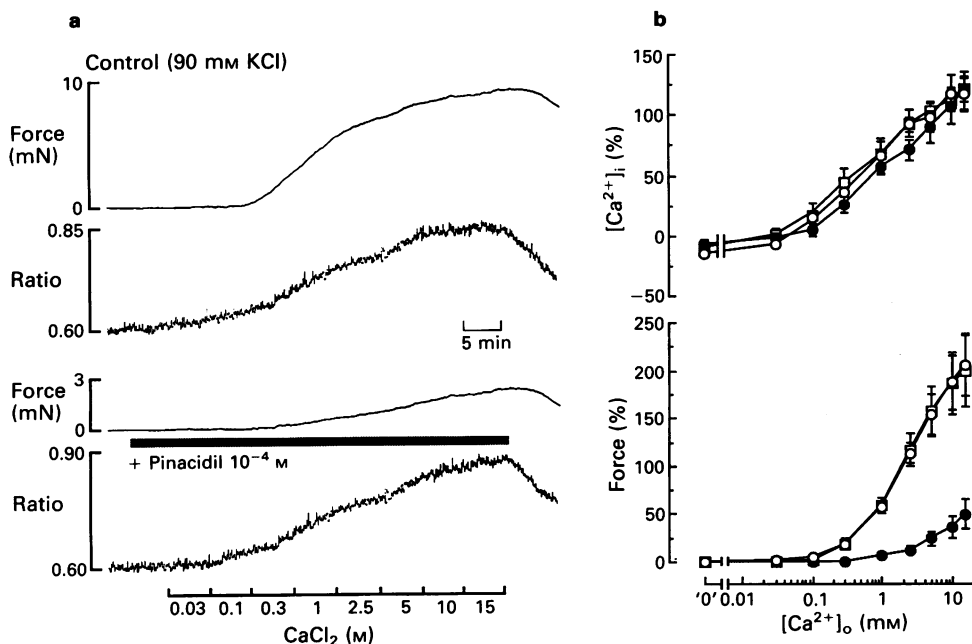


Figure 9 (a) Changes in [Ca²⁺]_i and force produced by increasing [Ca²⁺]_o in a canine coronary arterial muscle depolarized with 90 mM KCl-PSS and the effects of pinacidil on these changes. (b) Summarized data obtained from 6 experiments. Control (○), in the presence of 10⁻⁴ M pinacidil (●), washout (□).

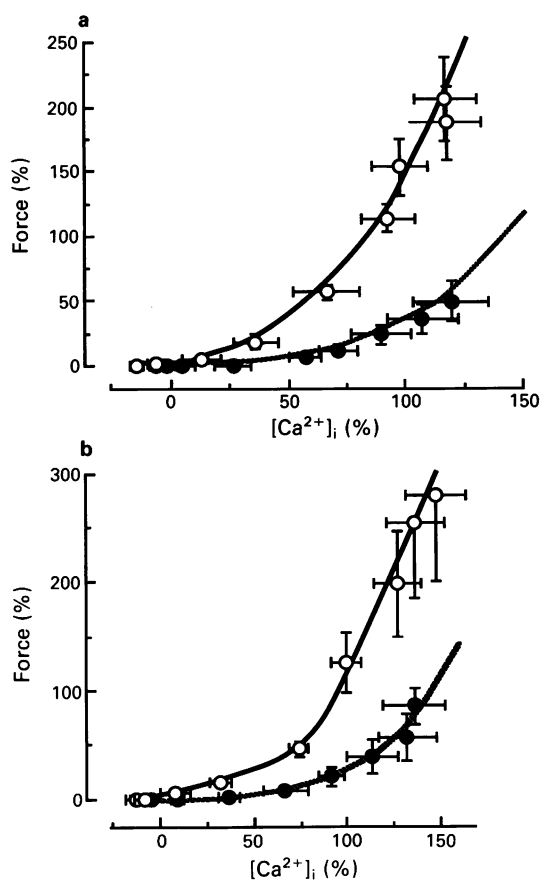


Figure 10 Relationship between $[Ca^{2+}]_i$ and force in the absence (control, ○), and the presence of 10^{-4} M pinacidil (a, ●) or 3×10^{-4} M nicorandil (b, ●). Vertical and horizontal bars are s.e.mean. The curves were fitted by eye.

in both normal and high KCl-PSS indicates that glibenclamide-sensitive K^+ channels are closed under these conditions.

In 30 mM KCl-PSS in which the coronary artery generated force, all three K^+ channel openers reduced both the force of contraction and the increase in $[Ca^{2+}]_i$. However, cromakalim reduced tension development by only 45%, whereas pinacidil and nicorandil abolished the force of contracture. The maximum % reduction of the increase in $[Ca^{2+}]_i$ by the three K^+ channel openers was in the range 36–45%. Although the reduction of the increase in $[Ca^{2+}]_i$ by the three K^+ channel openers and the relaxant effect of cromakalim were abolished

by 10^{-3} M TBA or 10^{-5} M glibenclamide, these K^+ channel blockers only partly attenuated the relaxant effects of pinacidil and nicorandil. In 45 and 90 mM KCl-PSS, cromakalim failed to affect the increase in $[Ca^{2+}]_i$ and force as has been shown in rat blood vessels (Weir & Weston, 1986b), whereas pinacidil and nicorandil relaxed the tissues without reducing the increase in $[Ca^{2+}]_i$. However, this increased $[Ca^{2+}]_i$ was reduced by 10^{-5} M verapamil to basal levels (Figure 8), suggesting that it was due to an influx of extracellular Ca^{2+} .

Nicorandil contains a nitroxy moiety within its structure and is known to increase intracellular levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) by activating soluble guanylate cyclase in various vascular smooth muscle tissues (Endoh & Taira, 1983; Holzmann, 1983) and in cardiac muscle (Yanagisawa *et al.*, 1988). The resultant activation of cyclic GMP-dependent protein kinase is thought to be responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). Thus, it is likely that nicorandil partly relaxes smooth muscle cells without reducing the increase in $[Ca^{2+}]_i$ following Ca^{2+} influx by depolarization, via mechanisms like those of nitroglycerin (Yanagisawa *et al.*, 1989). Unlike nicorandil, pinacidil does not change cyclic GMP levels in either vascular smooth muscle (Kauffman *et al.*, 1986) or in cardiac muscle (Yanagisawa *et al.*, 1988). Thus, pinacidil exerts some of its relaxant effects by a mechanism independent of the involvement of cyclic nucleotides but which remains to be determined.

The relaxant potencies of pinacidil and nicorandil in PSS containing KCl in concentrations of 45 mM or greater were reduced at the upper end of the KCl concentration range (45–90 mM) (Table 1). This phenomenon was due to the changed relationship between $[Ca^{2+}]_i$ and force observed in the presence of pinacidil or nicorandil (Figure 10). A similar rightward shift of the $[Ca^{2+}]_i$ -tension relationship by cyclic GMP has previously been obtained (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The mechanism involved is uncertain, but in the case of nicorandil the effects of an increase in $[Ca^{2+}]_i$ in generating force may have been counteracted by a cyclic GMP-mediated inhibition of myosin light chain phosphorylation.

This present study was supported by Grant-in-Aids for Special Project Research (No. 61132001), for Scientific Research (No. 62440027), for Developmental Scientific Research (No. 62870008) and for Encouragement of Young Scientist (No. 61770133) from the Ministry of Education, Science and Culture, Japan. We are also grateful to Dr T.C. Hamilton, Beecham Pharmaceuticals, The Pinnacles, Harlow, Essex, U.K. for the supply of cromakalim, to Shionogi & Co., Ltd., Osaka, Japan for pinacidil, to Chugai Pharmaceutical Co., Ltd., Tokyo, Japan for nicorandil and to Hoechst Japan Ltd., Tokyo, Japan and Yamanouchi Pharmaceutical Co., Tokyo, Japan for glibenclamide. The authors are grateful to Dr M.P. Kingsbury for his helpful improvement of this manuscript.

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(Received January 4, 1990)

Revised April 22, 1990

Accepted May 18, 1990