Nitroglycerin relaxes canine coronary arterial smooth muscle without reducing intracellular Ca^{2+} concentrations measured with fura-2

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1   Changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured simultaneously with force by a microfluorometric method using a calcium indicator, fura-2, in canine coronary arterial smooth muscle cells.

2   Depolarization by high (30–90 mM) KCl-physiological salt solution (PSS) produced concentration-dependent increases in force and $[Ca^{2+}]_i$.

3   The KCl-induced increase in $[Ca^{2+}]_i$ was abolished by Ca^{2+}-free conditions and almost abolished by verapamil 10^{-5} M, suggesting that it was entirely due to the increased Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

4   The $[Ca^{2+}]_i$-force relationship in the presence of verapamil was not distinguishable from that of control.

5   Nitroglycerin produced a concentration-dependent, reversible contraction of the coronary artery that had been contracted by high KCl-PSS, without reduction of the increased $[Ca^{2+}]_i$.

6   The KCl-induced increase in $[Ca^{2+}]_i$ was not affected by nitroglycerin and in the presence of nitroglycerin it was abolished by 10^{-5} M verapamil suggesting that it was caused by the influx of extracellular Ca^{2+}.

7   The $[Ca^{2+}]_i$-force curve was shifted to the right by nitroglycerin.

8   It is likely that nitroglycerin relaxes the coronary arterial smooth muscle by reducing the amount of myosin light chain phosphorylation even in the presence of raised $[Ca^{2+}]_i$ produced by increased Ca^{2+} influx following depolarization.

Introduction

Although nitroglycerin, the prototypical nitrate, has been used for more than a century, the subcellular mechanism of action of this class of vasodilators has become understood only in the last decade. Nitrates including nitroglycerin increase the amount of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) in smooth muscle tissues (Schultz *et al.*, 1977; Kukovetz *et al.*, 1979) by activating guanylate cyclase (Kimura *et al.*, 1975; Ignarro and Kadowitz, 1985). The activation of cyclic GMP-dependent protein kinase by increased cyclic GMP is thought to be primarily responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). Although the first intracellular mediator of nitrates has been identified as above, the final step directly responsible for vasodilatation remains to be elucidated. The candi-

dates for this step are membrane hyperpolarization (Ito *et al.*, 1978), inhibition of Ca^{2+} influx (Thorens & Haeusler, 1979; Karaki *et al.*, 1984), increase in Ca^{2+} extrusion (Itoh *et al.*, 1985), Ca^{2+} sequestration (Imai & Kitagawa, 1981; Dubé *et al.*, 1988) and inhibition of the contractile elements (Pfitzer *et al.*, 1984) in vascular smooth muscle. It seems essential to know the effect of nitroglycerin on $[Ca^{2+}]_i$ in order to test these possibilities. We have recently developed a microfluorometric method, using a Ca^{2+} indicator, fura-2 (Grynkiewicz *et al.*, 1985), to measure $[Ca^{2+}]_i$ simultaneously with force in isolated vascular rings. Fluorometric methods using fura-2 have been developed so as to record mechanical activity simultaneously (Ozaki *et al.*, 1987; Bruschi *et al.*, 1988). Using the new method we investigated whether nitroglycerin changes $[Ca^{2+}]_i$ simultaneously with its relaxant effect on isolated

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canine coronary arterial rings. We compared the effects of nitroglycerin with those of verapamil which is known to inhibit Ca^{2+} influx by blocking Ca^{2+} channels opened by depolarization (Godfraind *et al.*, 1986).

Methods

Hearts were excised 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 tissues were carefully removed in a dissecting chamber under a binocular microscope. Endothelium was removed by gentle rubbing and the luminal side was turned outwards. The composition (mmol litre^{-1}) of the physiological salt solution (PSS) was as follows: NaCl 140, KCl 5, CaCl$_2$ 2.5, MgCl$_2$ 2.5, glucose 11.1 and HEPES 3 (pH 7.4). The solution was equilibrated with 100% O$_2$ at 37°C. High K$^+$ solution was made by substituting NaCl with equimolar KCl. Ca^{2+}-free solution was made by removing CaCl$_2$ from the PSS. Coronary arterial rings were exposed to 10 μM fura-2 acetoxymethyl ester (fura-2 AM) for about 2 h at 37°C. The noncytotoxic detergent, Pluronic F-127, was premixed in the loading PSS (0.1%) to help solubilize 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.

Experiments were performed with a fluorometer using the dual wavelength excitation method (CAM-200, Japan Spectroscopic, Tokyo, Japan) specially designed to measure the fluorescence of muscle cells conjugated to the inverted microscope (TMD, Nikon, Tokyo, Japan) (Figure 1a). The excitation light was obtained from a xenon high-pressure lamp (150 W) equipped with a monochrometer. Ultraviolet light of alternating 340 and 380 nm (band width: 10 nm) was obtained by a chopping wheel (100 to 1000 Hz) placed in front of a monochrometer. To minimize the photobleaching of fura-2, the excitation light was reduced by an ND filter to 10% and the preparation was exposed to the excitation light intermittently (usually 2 s excitation and 8 s darkness) (Becker & Fay, 1987). The ratio of the fluoroscence due to excitation at 340 nm (F$_{340}$) to that at 380 nm (F$_{380}$) was calculated from successive illumination periods and referred to as the ratio (F$_{340}$/F$_{380}$). The fluorescence image, using a Nikon CF UV (Fluor) 10X, was obtained from the smooth muscle cells in the media layer by focusing on them. 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} (Figure 1b). 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 fluorescence signals, F$_{340}$, F$_{380}$ and their ratio, were sampled and held by using S/H amplifiers (SHM-IC-1, Datel, Tokyo, Japan). The photosignals and the mechanical activity were measured simultaneously and recorded on a chart recorder (Rectihoriz-8k, NEC-San-ei, Tokyo, Japan). The fluorescence signals and tension signal were also digitized by A/D converters and input into a personal computer (PC-9801, NEC, Tokyo, Japan). Lotus 1-2-3 (Lotus Development Co., Tokyo, Japan) was used for the calculation and graphical analysis (Himpens & Somlyo, 1988). Every experiment was started with perfusion with 90 mM KCl-PSS for 10 min. At the end of every experiment, external Ca^{2+} was removed and [Ca^{2+}]$_i$ was minimized by replacing PSS with 10 mM EGTA dissolved in Ca^{2+}-free PSS neutralized with HCl. The minimum ratio was obtained by this procedure. After removing the EGTA solution, 0.3 ml normal PSS containing 1% Triton X-100 was applied, by which procedure the maximum ratio was obtained. After adding 0.3 ml 10 mM EGTA solution, the fluorescence due to fura-2 was abolished by addition of 0.1 ml of 200 mM MnCl$_2$. The remaining fluorescence was regarded as autofluorescence whose intensity and ratio were very similar to that of unloaded muscle. We subtracted the corresponding value due to autofluorescence from F$_{340}$ and F$_{380}$ to derive a recalculated ratio$_{rc}$. Changes in ratio$_{rc}$ and force were expressed by taking the differences between basal values and those of the responses to 90 mM KCl-PSS at 10 min as 100%.

The following drugs and chemicals were used: nitroglycerin (Nippon Kayaku, Tokyo, Japan), verapamil hydrochloride (Eisai, Tokyo, Japan), EGTA (glycoletherdiaminetetraacetic acid), fura-2 AM, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and Pluronic F-127 (Dojin, Kumamoto, Japan). 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/w in DMSO. Other drugs were dissolved in distilled water in the desired concentrations.

The concentration-effect curves for verapamil and nitroglycerin were expressed as % reduction in the pre-drug values of force of contracture or increased [Ca^{2+}]$_i$ and computer fitted to a logistic equation:

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

where E is normalized response. A is drug concentration, K is EC$_{50}$ value of each drug and p is slope parameter (Parker & Waud, 1971).
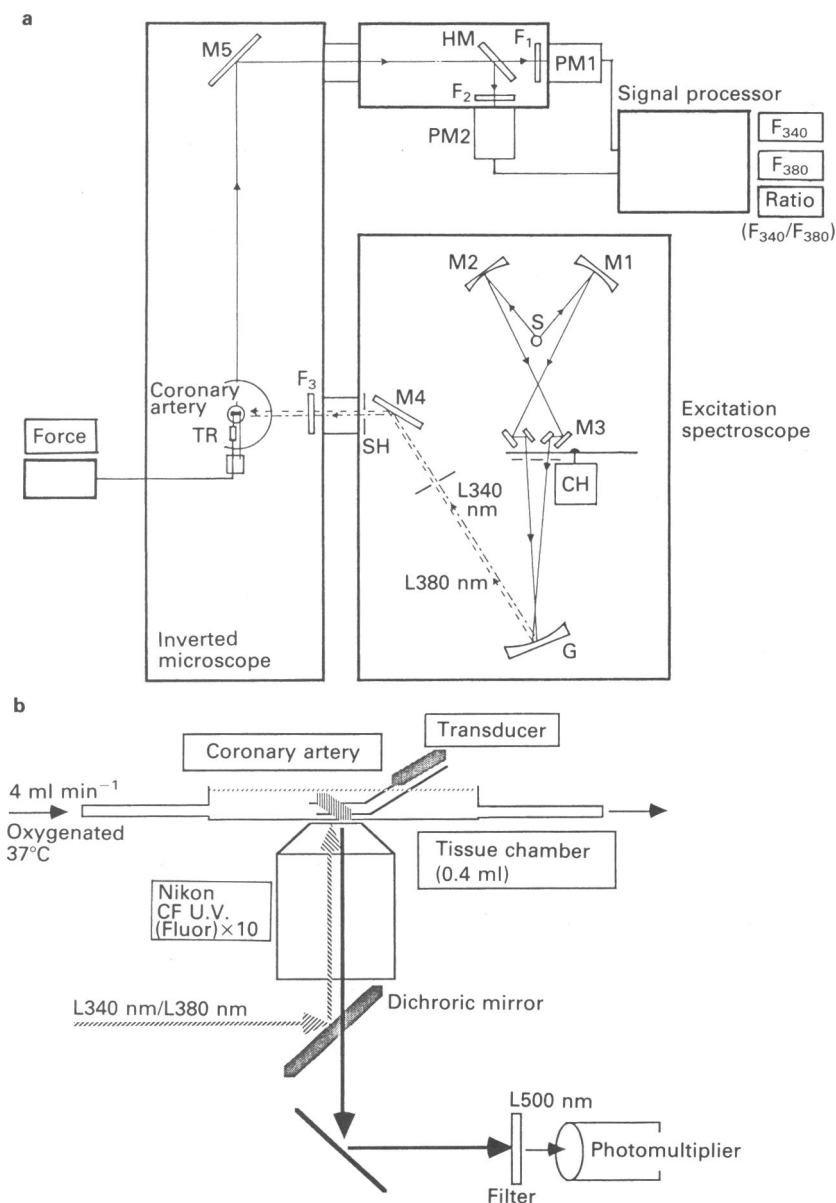
Figure 1   (a) A diagram of a microscopic fluorometer using the dual wavelength excitation method. The excitation light was obtained from a xenon high-pressure lamp (S) equipped with a monochrometer (G). Alternating u.v. lights of 340 and 380 nm (band width: 10 nm) were obtained by the chopping wheel (CH) placed in front of a monochrometer. To minimize photobleaching of fura-2, the excitation light was reduced by an ND filter (F3) to 10% and the preparation was exposed to the excitation light intermittently (electromechanical shutter: SH); 500 nm fluorescent light through a half mirror (HM) and a filter (F1) was obtained by a photomultiplier (PM1). Another photomultiplier (PM2) and a filter (F2) were used when the preparations were loaded with another Ca^{2+} indicator, Indo-1. The fluorescence due to excitation at 340 nm (F_{340}), that at 380 nm (F_{380}) and the ratio (F_{340}/F_{380}) were sampled and held by using S/H amplifiers and continuously recorded on a chart recorder and digitized and input into a personal computer. (b) The fluorescence image, using a Nikon CF U.V. (Fluor) 10X, was obtained from the smooth muscle cells in the media layer by focusing on them. 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 $4\, ml\, min^{-1}$.

Table 1 Changes in force of contracture, ratio$_{rc}$ and $[Ca^{2+}]_i$ induced by KCl-PSS in canine coronary artery

KCl (mM)	n	Force (mN)	Force (%)	Ratio	$[Ca^{2+}]_i$ (%)
5	23	0	0	0.90 ± 0.05	0
30	11	1.71 ± 0.25	46.8 ± 4.1	1.08 ± 0.07	73.3 ± 6.1
45	9	3.40 ± 0.46	67.8 ± 11.5	1.14 ± 0.12	80.1 ± 15.1
60	12	2.57 ± 0.31	92.8 ± 9.5	1.18 ± 0.10	88.4 ± 8.2
90	23	3.81 ± 0.34	100	1.20 ± 0.06	100

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

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

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

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

Results

Changes in mechanical activity and $[Ca^{2+}]_i$ induced by depolarization with high KCl-PSS in canine coronary artery

In fura-2 unloaded muscles, 90 mM KCl-PSS caused an increase in tension with concomitant slight increases in F_{340} and F_{380}, but the ratio was not changed, because the increases in F_{340} and F_{380} were proportional (Figure 2a). F_{340} and F_{380} increased more slowly than did tension but the maxima of F_{340} and F_{380} occurred at the time when tension reached maximum. Thus, even if movements of muscles influence F_{340} and F_{380}, this movement artifact could be circumvented by taking their ratio (Sato *et al.*, 1988). Basal F_{340} and F_{380} increased 2-4 times higher in fura-2 loaded muscles than in unloaded muscles, and decayed at a rate of 0.1-0.4% per min; 90 mM KCl-PSS elevated tension with a rapid increase in F_{340} but a decrease in F_{380}. Thus, the ratio was increased and the peak of the increment was attained at about 1 min after perfusion with 90 mM KCl-PSS. The ratio remained high during the high KCl-induced contracture which tended to increase with time (Figure 2b). In canine coronary artery, depolarization produced by 20 mM KCl-PSS slightly increased $[Ca^{2+}]_i$ but it produced no contracture. The threshold increase in $[Ca^{2+}]_i$
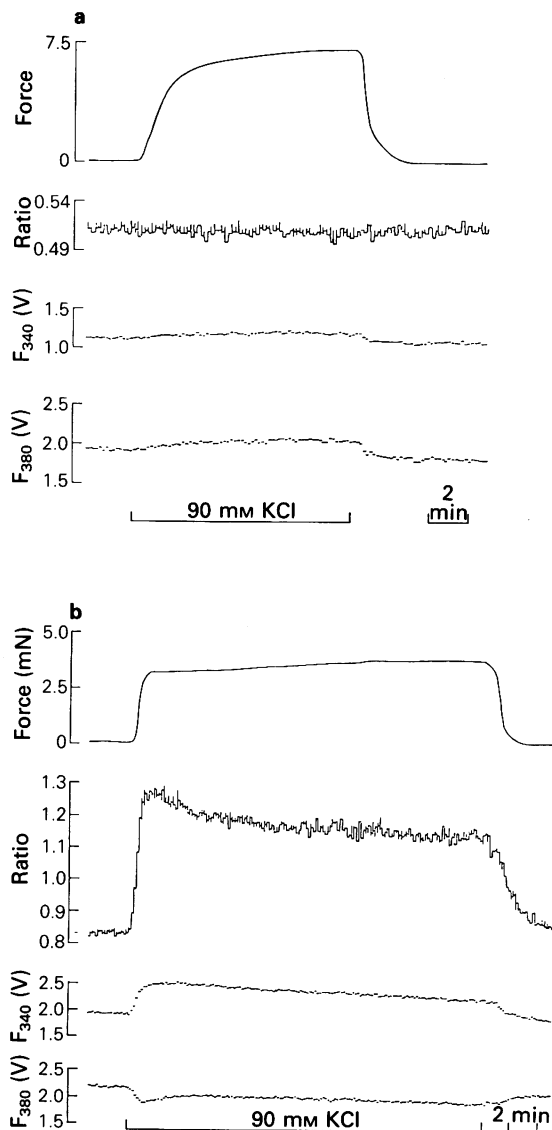

Figure 2 Typical recordings of changes in fluorescence and force development produced by 90 mM KCl measured simultaneously in a coronary arterial muscle without (a) or with (b) fura-2 loading.
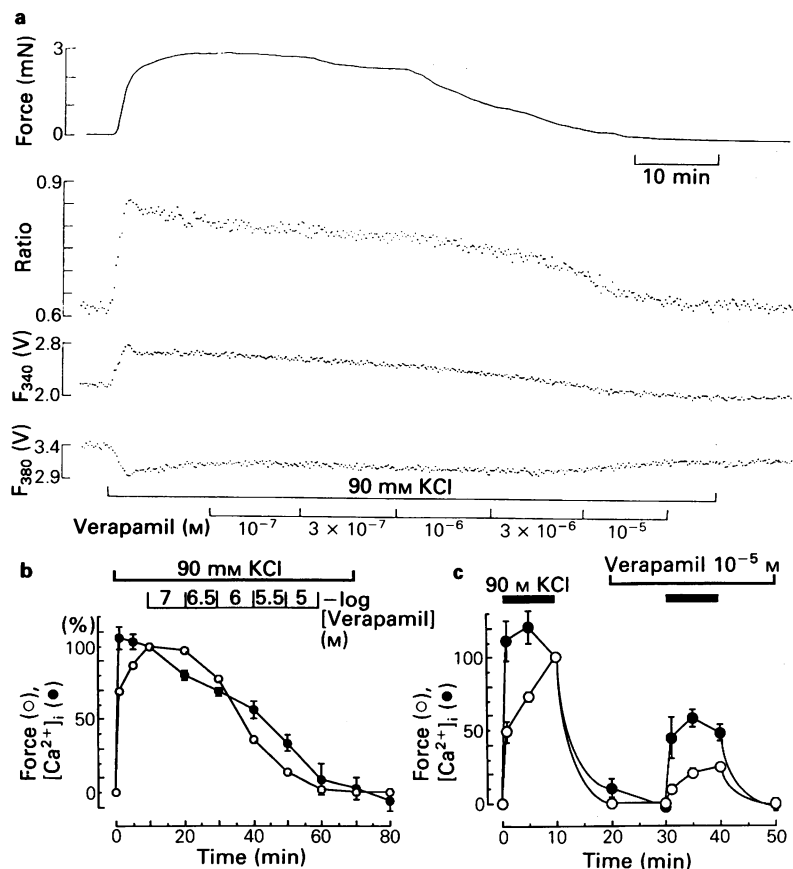
Figure 3 (a) Effects of verapamil (10^{-7}–10^{-5} M) on force and changes in fluorescence produced by 90 mM KCl-PSS in a canine coronary artery. (b) Summarized data which were normalized in relation to the values of 90 mM KCl at 10 min ($n = 6$). (c) Effects of 10^{-5} M verapamil on the basal force and $[Ca^{2+}]_i$ (5 mM KCl-PSS) and 90 mM KCl-induced changes in $[Ca^{2+}]_i$ and force ($n = 5$).

required to generate force was 10–20%. The increase in $[Ca^{2+}]_i$ was less than 10% and the reduction of $[Ca^{2+}]_i$ by reducing $[Ca^{2+}]_o$ in canine coronary artery did not change the force. Table 1 shows changes in $[Ca^{2+}]_i$ and force of contracture produced by 30–90 mM KCl-PSS at 10 min normalized to the effects by 90 mM KCl-PSS.

Effect of verapamil on KCl-induced changes in mechanical activity and $[Ca^{2+}]_i$ in canine coronary artery

Figure 3a shows the effect of verapamil on 90 mM KCl-PSS-induced changes in mechanical activity and $[Ca^{2+}]_i$ signal in canine coronary artery. Verapamil (10^{-7}–10^{-5} M) reduced in a concentration-dependent manner both force and $[Ca^{2+}]_i$ which had been increased by 90 mM KCl-PSS. The results

of analysis of the concentration-effect curves for verapamil to reduce the contracture and the increased $[Ca^{2+}]_i$ due to 45 mM KCl-PSS are summarized in Table 2. The pD_2 ($-\log EC_{50}$) values of verapamil producing both effects were not different at one concentration of KCl, although the slope parameters of both curves were different. Interestingly, these values of verapamil were also not different between 45 mM and 90 mM KCl in spite of the difference of the degree of membrane depolarization. When the muscle was exposed to 10^{-6} M verapamil, the basal $[Ca^{2+}]_i$ tended to decrease, although the basal force was not changed. However the slight reduction of $[Ca^{2+}]_i$ by verapamil could not be distinguished from the gradual decline of $[Ca^{2+}]_i$ following a previous challenge. The increased $[Ca^{2+}]_i$ and force of contracture due to 90 mM KCl-PSS were decreased by 10^{-6} M verapamil to 47.8 ± 4.3% and
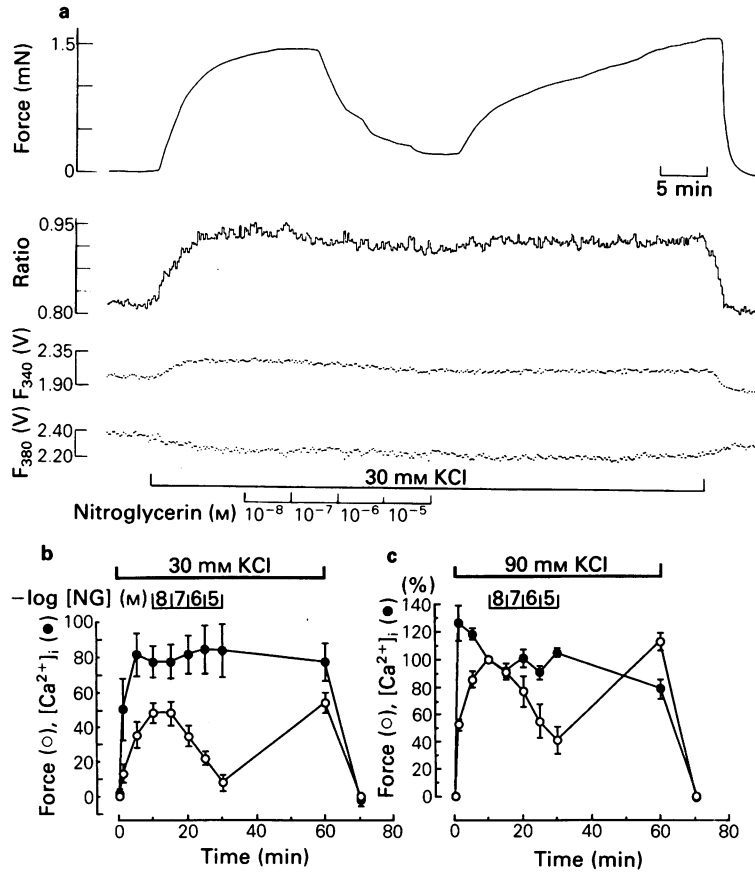
Figure 4 (a) Effects of nitroglycerin (NG) (10^{-8}–10^{-5} M) on the 30 mM KCl-PSS induced changes in $[Ca^{2+}]_i$ and force. The effects of nitroglycerin could be reversibly washed out by 30 min perfusion with 30 mM KCl-PSS. (b) Summarized data obtained from 6 experiments with 30 mM KCl-PSS. (c) Summarized data obtained from 5 experiments with 90 mM KCl-PSS.

Table 2 Effects of verapamil and nitroglycerin in reducing the force of contracture and the increase in $[Ca^{2+}]_i$ induced by KCl

		Reduction of force		Reduction of $[Ca^{2+}]_i$	
KCl (mM)	n	pD$_2$	Slope parameter (p)	pD$_2$	Slope parameter (p)
Verapamil					
45	5	6.26 ± 0.06	1.45 ± 0.15	6.34 ± 0.24	0.87 ± 0.10
90	5	6.15 ± 0.08	1.47 ± 0.18	5.99 ± 0.16	0.72 ± 0.28
Nitroglycerin					
30	6	6.25 ± 0.19	0.65 ± 0.06	No effect ($< 10^{-5}$ M)	
60	6	5.54 ± 0.10*	0.62 ± 0.09	No effect ($< 10^{-5}$ M)	
90	5	5.35 ± 0.17*	0.54 ± 0.06	No effect ($< 10^{-5}$ M)	

The concentration-effect curves for verapamil and nitroglycerin were analysed by computer fitting to a logistic equation: $E = 100 - 100 \times A^p/(A^p + K^p)$ where E is normalized response, A is drug concentration, K is EC$_{50}$ value of each drug and p is the slope parameter. EC$_{50}$ values were presented as pD$_2$ (pD$_2$ = $-\log$ EC$_{50}$). *$P < 0.05$ compared with 30 mM KCl.
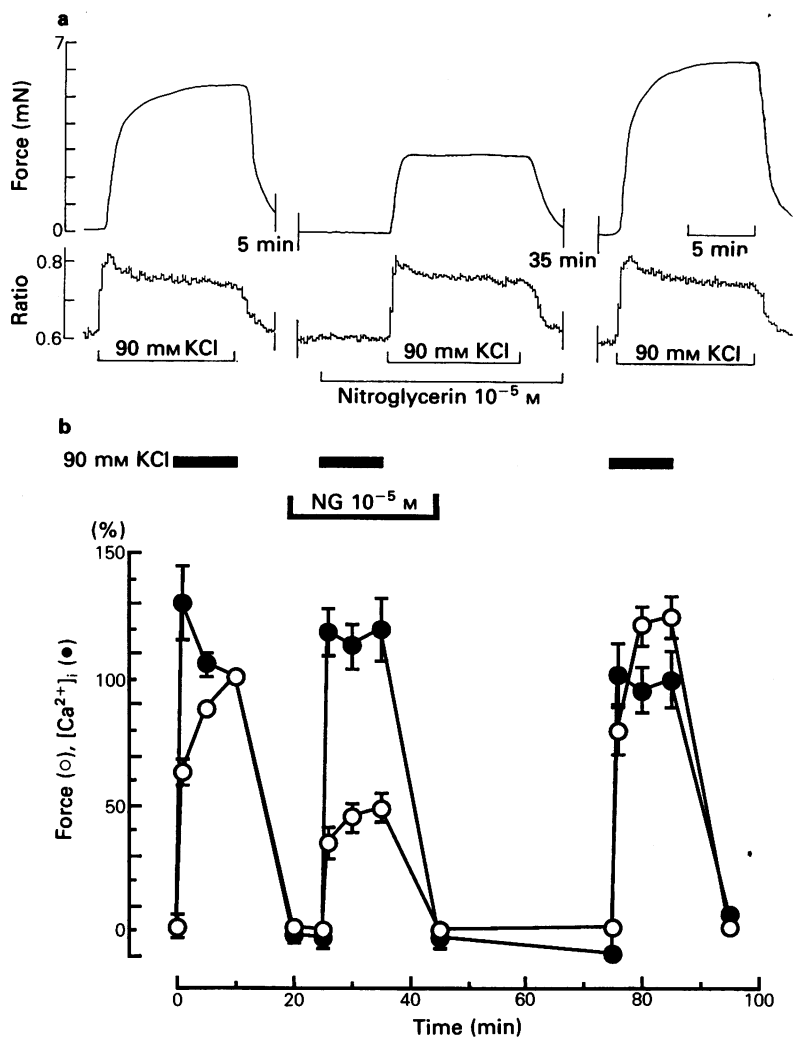
Figure 5 (a) Effects of 10^{-5} M nitroglycerin (NG) on the basal force and $[Ca^{2+}]_i$ (5 mM KCl-PSS) and 90 mM KCl-PSS induced changes in $[Ca^{2+}]_i$ and force. The effect of nitroglycerin on force of contracture was washed out by perfusion with normal PSS. (b) Summarized data obtained from 7 experiments.

23.8 ± 7.9%, respectively ($n = 5$) (Figure 3b). Similar reductions were produced by 10^{-6} M verapamil of the contracture previously induced by 90 mM KCl-PSS (Figure 3c).

Effect of nitroglycerin on KCl-induced changes in mechanical activity and $[Ca^{2+}]_i$ in canine coronary artery

Figure 4a shows the effect of nitroglycerin on force of contracture and fluorescence signals from fura-2

induced by 30 mM KCl-PSS in a canine coronary artery. Nitroglycerin (10^{-8}–10^{-5} M) reduced the force of contracture in a concentration-dependent manner, but did not affect $[Ca^{2+}]_i$ at all. The effect of nitroglycerin on the force of contracture could be reversibly washed out by perfusion with 30 mM KCl-PSS. Even during the washout of nitroglycerin $[Ca^{2+}]_i$ did not change at all. These results indicate that, in canine coronary artery, nitroglycerin relaxes the KCl-PSS-induced contracture without reducing $[Ca^{2+}]_i$. Summarized data of force and $[Ca^{2+}]_i$ are
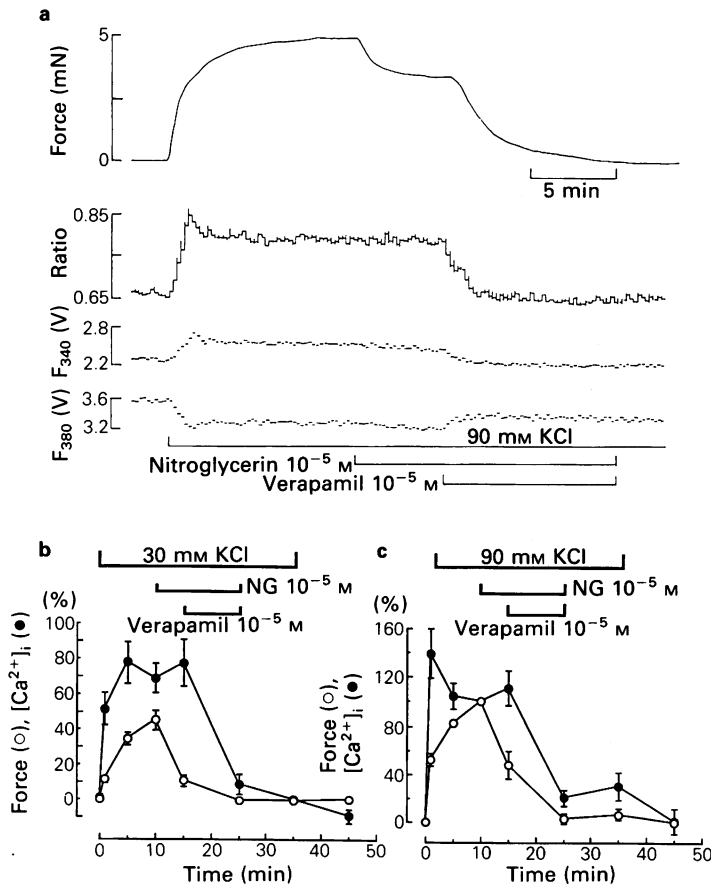
Figure 6   (a) Effects of 10^{-5} M nitroglycerin (NG) and 10^{-5} M verapamil on changes in $[Ca^{2+}]_i$ and force of contracture produced by 90 mM KCl-PSS. The $[Ca^{2+}]_i$ which remained increased in the presence of nitroglycerin was reduced to the basal level by the application of 10^{-5} M verapamil. (b) Summarized data obtained from 5 experiments with 30 mM KCl-PSS. (c) Summarized data obtained from 6 experiments with 90 mM KCl-PSS.

shown in Figure 4b (30 mM KCl-PSS) and 4c (90 mM KCl-PSS) and analysed data of the concentration-response curves for nitroglycerin to reduce the force of contracture are also shown in Table 2. The potency of nitroglycerin in causing relaxation was dependent on KCl concentrations, e.g., the pD_2 values of nitroglycerin became greater when the muscle was depolarized to a smaller extent or when increases in $[Ca^{2+}]_i$ in muscles were smaller. Figure 5 shows the effect of nitroglycerin on the basal force and $[Ca^{2+}]_i$ and the force of contracture and the increased $[Ca^{2+}]_i$ due to 90 mM KCl-PSS. Nitroglycerin (10^{-5} M) did not change basal $[Ca^{2+}]_i$. Although it reduced the force of contracture induced by 90 mM KCl-PSS, the increased $[Ca^{2+}]_i$ was not affected at all. The reduction in force produced by

nitroglycerin could also be reversibly washed out by perfusion with normal PSS.

Effect of verapamil on the increase in $[Ca^{2+}]_i$ induced by high KCl-PSS in the presence of nitroglycerin in canine coronary artery

The results showing that nitroglycerin relaxed the coronary artery contracted by KCl without changing $[Ca^{2+}]_i$ were so interesting that we examined whether the increased $[Ca^{2+}]_i$ due to high KCl-PSS which remained unaffected by nitroglycerin is susceptible to verapamil, as was the case with the absence of nitroglycerin. Figure 6 shows such an experiment and summarized data for 30 mM or

90 mM KCl-PSS. Without changing $[Ca^{2+}]_i$, nitroglycerin (10^{-5} M) reduced the force of contracture induced by 30 mM and 90 mM KCl-PSS to $22.4 \pm 6.3\%$ and $47.9 \pm 11.6\%$ (force produced by 30 mM or 90 mM KCl-PSS at 10 min = 100%), respectively. Perfusion with KCl-PSS containing both 10^{-5} M nitroglycerin and 10^{-5} M verapamil abolished the remaining force and the increased $[Ca^{2+}]_i$. Thus, the result that $[Ca^{2+}]_i$ remained increased in the presence of nitroglycerin but was reduced by verapamil almost to the basal level indicates that the increased $[Ca^{2+}]_i$ was derived from the influx of Ca^{2+} through Ca^{2+} channels opened by KCl-PSS-induced depolarization and that nitroglycerin can relax the coronary artery without reducing $[Ca^{2+}]_i$.

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

As described in the previous section, the relaxant potency of nitroglycerin was dependent on KCl concentrations, e.g., the pD_2 values of nitroglycerin were greater when the muscle was depolarized to a smaller extent or when the increases in $[Ca^{2+}]_i$ in muscles were smaller (Table 2). To investigate whether the degree of depolarization or the increase in $[Ca^{2+}]_i$ influences the relaxant potency of nitroglycerin, coronary muscle was at first perfused with Ca^{2+}-free PSS for 10 min and then with Ca-free 90 mM KCl-PSS. Removing extracellular Ca^{2+} produced a reduction of $[Ca^{2+}]_i$ ($-27 \pm 2\%$), whereas the force was not changed. After removal of extracellular Ca^{2+} by perfusion with Ca^{2+}-free PSS, depolarization of the muscle by 90 mM KCl failed to produce any increase in $[Ca^{2+}]_i$ ($-31 \pm 2\%$) or any change in force. In the presence of 90 mM KCl-PSS, the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was increased from 0.03 to 15 mM. As $[Ca^{2+}]_o$ was increased, $[Ca^{2+}]_i$ was also increased from 0.03 mM $[Ca^{2+}]_o$ and force was generated at 0.1 mM $[Ca^{2+}]_o$;

both $[Ca^{2+}]_i$ and force became saturated at 15 mM $[Ca^{2+}]_o$ (Figure 7). The relation between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ shows that of $[Ca^{2+}]_o$ and Ca^{2+} influx through voltage-sensitive Ca^{2+} channels in vascular smooth muscle. The results of computer fitting of the curve to the logistic equation are summarized in Table 3. The maximum is the difference between values at $[Ca^{2+}]_o$-free and 15 mM $[Ca^{2+}]_o$. The pD_2 values for $[Ca^{2+}]_o$ of 3.19 ± 0.13 indicate the apparent K_D values of $[Ca^{2+}]_o$ for the binding constant of Ca^{2+} channel opened by depolarization. The relationship between $[Ca^{2+}]_o$ and force of contracture was also analysed and is summarized in Table 3. The maximum force produced by KCl depolarization was $225 \pm 44\%$ and pD_2 values for $[Ca^{2+}]_o$ were 2.73 ± 0.05 and the slope parameter was 1.17 ± 0.04. The difference in pD_2 values for $[Ca^{2+}]_o$ between increase in $[Ca^{2+}]_i$ and increase in force seemed to be due to the steepness of the curve of $[Ca^{2+}]_i$-force relationship (Figure 8).

When nitroglycerin 10^{-5} 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 $[Ca^{2+}]_i$, the force produced by the increased $[Ca^{2+}]_i$ was much smaller than that of control and the threshold values of $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ to generate force were increased (Figure 7). The pD_2 values in the presence of nitroglycerin 10^{-5} M, the maximum force and the pD_2 values for $[Ca^{2+}]_o$ were significantly reduced. Since the relationship between $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ was not changed by nitroglycerin, in the 90 mM KCl-PSS the membrane potential may have not been changed by nitroglycerin. Figure 7 also shows the relaxant effect of nitroglycerin was larger in low concentrations of $[Ca^{2+}]_o$ than in high concentrations. The effect of nitroglycerin was reversibly washed out and the muscle responded to an increase in $[Ca^{2+}]_o$ in 90 mM KCl-PSS as in the control (Figure 7 and Table 3). Thus, these results clearly show that nitroglycerin does not affect Ca^{2+} influx through Ca^{2+} channels, that its relaxant effect is operative without

Table 3 Effects of nitroglycerin (NG) on the $[Ca^{2+}]_o$-force and $[Ca^{2+}]_o$-$[Ca^{2+}]_i$ relationship in 90 mM KCl-depolarized canine coronary artery

| | | Increase in force | | | Increase in $[Ca^{2+}]_i$ | |
	Max (%)	pD_2	p (Slope parameter)	Max (%)	pD_2	p (Slope parameter)
Control	225 ± 44	2.73 ± 0.05	1.17 ± 0.04	190 ± 37	3.19 ± 0.13	0.83 ± 0.19
NG 10^{-5} M	$155 \pm 32^*$	$2.32 \pm 0.04^*$	1.19 ± 0.04	189 ± 29	3.23 ± 0.09	1.00 ± 0.16
Washout	251 ± 61	2.83 ± 0.01	1.12 ± 0.01	174 ± 30	3.17 ± 0.10	0.81 ± 0.22

Increase in $[Ca^{2+}]_i$ was calculated from the difference between that obtained by 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.
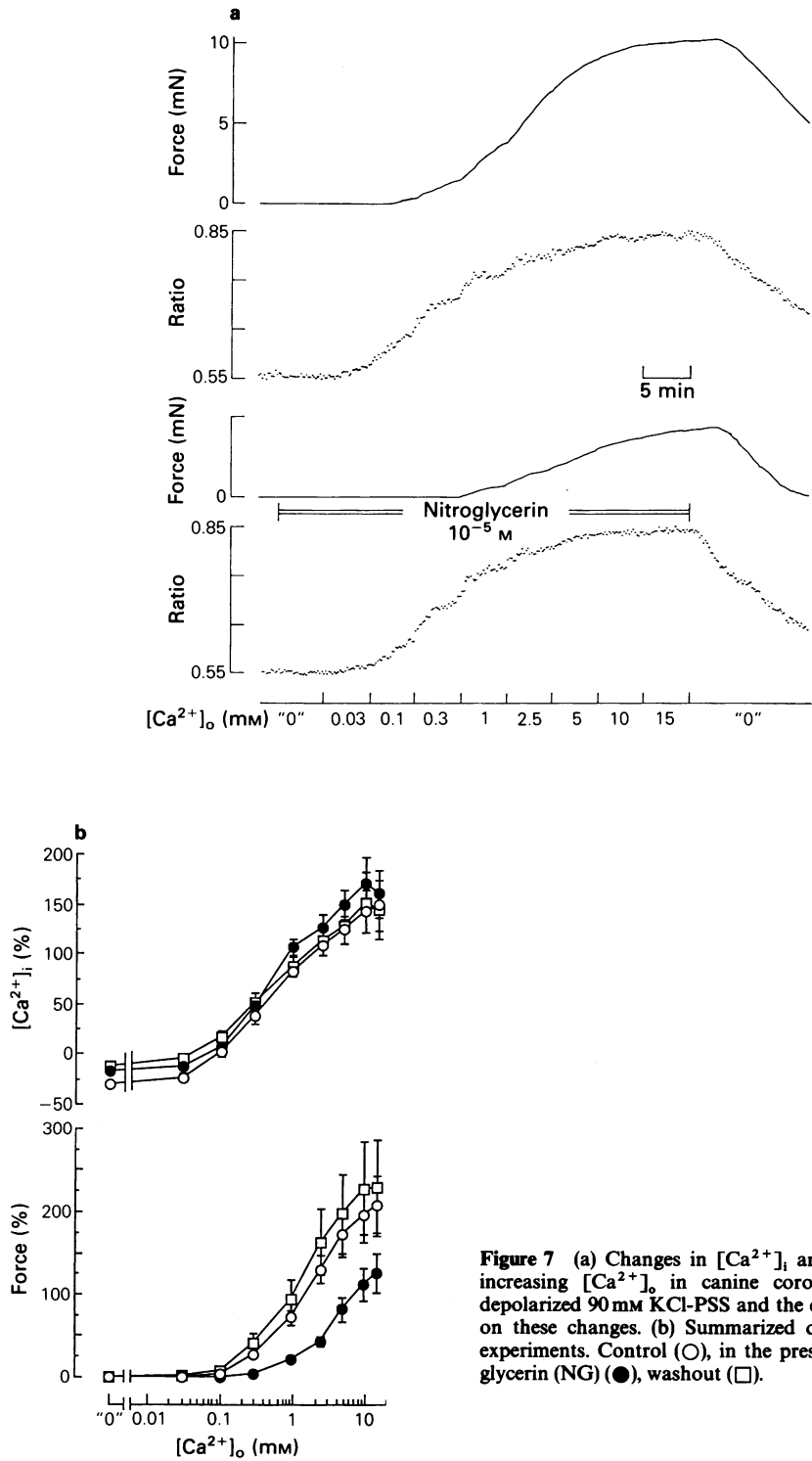
Figure 7 (a) Changes in $[Ca^{2+}]_i$ and force produced by increasing $[Ca^{2+}]_o$ in canine coronary arterial muscle depolarized 90 mM KCl-PSS and the effects of nitroglycerin on these changes. (b) Summarized data obtained from 6 experiments. Control (○), in the presence of 10^{-5} M nitroglycerin (NG) (●), washout (□).

reducing $[Ca^{2+}]_i$ and that the relaxant potency of nitroglycerin is dependent on $[Ca^{2+}]_i$ but not on the degree of depolarization.

Effects of nitroglycerin and verapamil on the relationship between $[Ca^{2+}]_i$ and force in depolarized canine coronary artery

Figure 8 shows the relationship between $[Ca^{2+}]_i$ and force in the absence and the presence of nitroglycerin (10^{-5} M) or verapamil in depolarized canine coronary artery. The relationship between $[Ca^{2+}]_i$ and force obtained by changing the degree of depolarization by 30, 45, 60 and 90 mM KCl (Table 1) was similar to that produced by changing $[Ca^{2+}]_o$ in 90 mM KCl-depolarized muscles (control). The curve in the presence of verapamil (10^{-7}–10^{-5} M), which was constructed from the data obtained from 45 and 90 mM KCl-depolarized coronary muscles (Figure 3b), was not different from that in the absence of the drugs (control). The curve in the presence of nitroglycerin (10^{-5} M) was different from that of control and it was shifted to the right.

Discussion

In the present study, we were able to measure changes in cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) simultaneously with force in isolated canine coronary arterial rings. Measurement of $[Ca^{2+}]_i$ in smooth muscle cells has been successful with quin-2 (Kobayashi et al., 1985; Williams & Fay, 1986). Simultaneous measurement of $[Ca^{2+}]_i$ with force has been performed in vascular smooth muscle by use of fura-2 (Ozaki et al., 1987; Bruschi et al., 1988). In the present study we measured $[Ca^{2+}]_i$ microfluorometrically using the dual wavelength excitation method. There are several advantages to this method (Yagi et al., 1988). First, we were able to observe smooth muscle cells through a microscope and obtain the fluorescence signals from the cells by focusing on them. Second, the simultaneous measurement of $[Ca^{2+}]_i$ with force allowed us to determine the precise relationship between $[Ca^{2+}]_i$ and force in the same coronary artery. Third, in the present system, the reduction of fluorescence signal due to photobleaching of fura-2 (Becker & Fay, 1987) was minimized by the attenuation of excitation intensity and by giving the excitation only while collecting data. This enabled us to run the same muscle for a long period, e.g., we were able to examine the washout effect of nitroglycerin. Fourth, the fluorescence signals were not disturbed by the extracellular leakage of fura-2, which was removed, if any occurred, by continuous perfusion of the tissue bath.

Although we were unable to determine absolute values of $[Ca^{2+}]_i$ because of a relatively large amount of autofluorescence (about 25–50% in fura-2 loaded preparations) and uncertainty of absolute concentrations of fura-2 in the smooth muscle, the semiquantification of $[Ca^{2+}]_i$ could be performed by % changes in ratio, normalized by the difference between the basal value and the 90 mM KCl-induced value.

In canine coronary artery, depolarization induced by 30–90 mM KCl-PSS produced KCl concentration dependent increases in $[Ca^{2+}]_i$ and force. The threshold increase in $[Ca^{2+}]_i$ to generate force was 10–20%. An increase in $[Ca^{2+}]_i$ of less than 10% and reduction of $[Ca^{2+}]_i$ by reducing $[Ca^{2+}]_o$ in canine coronary artery did not change the force. These results indicate that the basal force is due to the passive force generated by the elastic component of tissue but not due to the active force generated by smooth muscle, and that there is an influx of Ca^{2+} into smooth muscle cells by its electrochemical gradient even in the resting condition. The relationship between $[Ca^{2+}]_i$ and force obtained by changing the degree of depolarization by 30, 45, 60 and 90 mM KCl (Table 1) was similar to that obtained by changing $[Ca^{2+}]_o$ in 90 mM KCl-depolarized muscles. The increase in $[Ca^{2+}]_i$ produced by depolarization seems to be entirely due to the increased influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, since depolarization of smooth muscle which had been perfused with Ca^{2+}-free PSS failed to induce any increase in $[Ca^{2+}]_i$ and the increased $[Ca^{2+}]_i$ was reduced to the basal level by 10^{-5} M verapamil which is known to block the voltage-dependent Ca^{2+} channels (Godfraind et al., 1986). This result is different from that obtained in cultured smooth muscle cells of rat aorta in which depolarization induced release of Ca^{2+} from an intracellular storage site (Kobayashi et al., 1985), and from that in smooth muscle of guinea-pig aorta in which ryanodine reduced $[Ca^{2+}]_o$-induced contraction related to KCl-depolarization (Ito et al., 1986). Although in aortic smooth muscle cells, KCl-depolarization may have released Ca^{2+} from its storage sites, the KCl-depolarization-induced contraction has been commonly thought to be the result of influx of extracellular Ca^{2+} and not the release of sequestered Ca^{2+} (Kuriyama et al., 1982).

Verapamil (10^{-7}–10^{-5} M) reduced the increased force and $[Ca^{2+}]_i$ due to 45 or 90 mM KCl-PSS in a concentration-dependent manner. Although the pD_2 values for verapamil to reduce $[Ca^{2+}]_i$ and force were not different from each other at each KCl concentration, the slope parameter (p) for reduction of $[Ca^{2+}]_i$ was smaller than that at force. This result seems to be due to the steepness of $[Ca^{2+}]_i$-force relationship observed in various skinned smooth
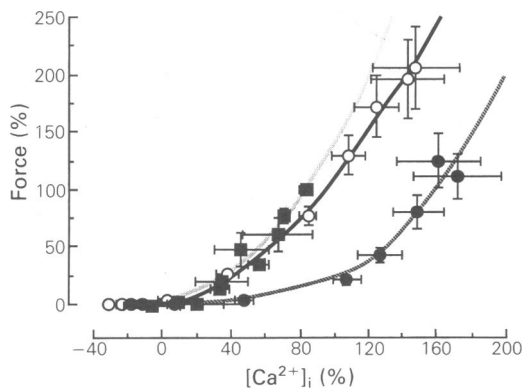
Figure 8 Relationship between $[Ca^{2+}]_i$ (%) and force (%) in the absence (control, ○), and in the presence of 10^{-5} M nitroglycerin (●) or 10^{-8}–10^{-5} M verapamil (■). Each point in the absence and presence of nitroglycerin was adopted from Figure 7(b) and that in the presence of verapamil from the experiments with 45 and 90 mM KCl. Vertical and horizontal bars are s.e.mean. Curves are 3 order polynomial least squares regression fits ($Y = a_1 X + a_2 X^2 + a_3 X^3 + b$). The curve in the presence of verapamil was not different from the control, whereas that in the presence of nitroglycerin was distinctly different from the control.

muscle preparations (Iino, 1981 for guinea-pig taenia coli; Itoh *et al.*, 1983; 1985 for rabbit mesenteric artery). When the pD_2 values were compared among different KCl concentrations, those obtained from 45 mM KCl-depolarized muscles were slightly but not significantly larger than those from 90 mM KCl. In the present study, the $[Ca^{2+}]_i$-force relationship in the presence of verapamil was not distinguishable from that of control. Thus, the relaxant effect of verapamil was entirely due to its well-known Ca^{2+} channel blocking effect (Godfraind *et al.*, 1986), without any influence on Ca^{2+} sensitivity of myofibrils.

Unlike verapamil, nitroglycerin relaxed coronary artery contracted by high KCl-PSS without reducing the increased $[Ca^{2+}]_i$. The relaxant effect of nitroglycerin was concentration-dependent and reversible (Figure 4). In the presence of nitroglycerin, $[Ca^{2+}]_i$ was not changed at all, whereas the increased $[Ca^{2+}]_i$ was reduced by 10^{-5} M verapamil to the basal level (Figure 6). Thus, the increased $[Ca^{2+}]_i$ which was unaffected in the presence of nitroglycerin was due to the influx of extracellular Ca^{2+}. Nitroglycerin failed to affect the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels (Figure 7). Thus, it is highly likely that nitroglycerin interfered with the genesis of force by $[Ca^{2+}]_i$.

Nitrates including nitroglycerin increase the amount of cyclic GMP in various vascular smooth muscle tissues (Schultz *et al.*, 1977; Kukovetz *et al.*, 1979) by activating guanylate cyclase (Kimura *et al.*, 1975; Ignarro & Kadowitz, 1985). The activation of cyclic GMP-dependent protein kinase is thought to be responsible for the vasodilator effect of nitrates (Waldman & Murad, 1987). It has been suggested that the final mechanism of action of organic nitrates for vasodilatation is hyperpolarization of the membrane (Ito *et al.*, 1978), inhibition of Ca^{2+} influx (Thorens & Haeusler, 1979; Karaki *et al.*, 1984), increase in Ca^{2+} extrusion (Itoh *et al.*, 1985), Ca^{2+} sequestration (Imai & Kitagawa, 1981; Dubé *et al.*, 1988) or inhibition of contractile elements (Pfitzer *et al.*, 1984). When the canine coronary arterial muscle was exposed to KCl-PSS, the depolarized membrane potential was not affected by nitroglycerin (Ito *et al.*, 1980). Thus, it is unlikely that hyperpolarization is responsible for the vasodilator effect of nitroglycerin in canine coronary artery. Although in aortic smooth muscle cells nitrates (Kobayashi *et al.*, 1985; Karaki *et al.*, 1988) and 8-bromo cyclic GMP (Kai *et al.*, 1987) reduce $[Ca^{2+}]_i$ by stimulation of Ca^{2+} extrusion via the Ca pump through activation of cyclic GMP-dependent protein kinase (Popescu *et al.*, 1985; Furukawa *et al.*, 1988), in KCl-induced depolarized canine coronary artery, $[Ca^{2+}]_i$ measured by fura-2 was not changed at all by nitroglycerin in all experiments while the relaxation was observed. Although the Ca^{2+} releasable storage sites, probably the sarcoplasmic reticulum (SR), may be developed in rat aorta and its cultured cells, in canine coronary artery they may be sparse. Thus, the vasodilator mechanism must be considered to be due to $[Ca^{2+}]_i$-independent mechanisms. There are several lines of evidence to support this supposition. In rat aorta, sodium nitroprusside relaxes KCl-induced contraction accompanied by a small decrease in $[Ca^{2+}]_i$ and a small decrease in ^{45}Ca uptake (Karaki *et al.*, 1988). In skinned porcine coronary artery, cyclic GMP inhibits contraction at intermediate $[Ca^{2+}]_i$ concentrations (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The amount of myosin light chain phosphorylation was reduced by sodium nitroprusside and 8-bromo cyclic GMP (Rapoport *et al.*, 1982; Draznin *et al.*, 1986). The phosporylation of myosin light chain which is activated by its kinase via Ca^{2+} and calmodulin complex is correlated with the amount of tension (Kamm & Stull, 1985). Thus, it is likely that nitroglycerin relaxes smooth muscle cells by reducing the amount of myosin light chain phosphorylation even in the presence of increased $[Ca^{2+}]_i$ following Ca^{2+} influx by depolarization.

The relaxant potency of nitroglycerin is known to be reduced by higher concentrations of KCl (see Ito *et al.*, 1980; Itoh *et al.*, 1983) (Table 2). This phenomenon seems to be due to the relationship between $[Ca^{2+}]_i$ and force in the presence of nitroglycerin

being shifted to the right (Figure 8). The rightward shift of $[Ca^{2+}]_i$-tension relationship by cyclic GMP has also been reported (Pfitzer *et al.*, 1984; Itoh *et al.*, 1985). The inhibitory effect of nitroglycerin on myosin light chain phosphorylation may be counteracted by the increase in $[Ca^{2+}]_i$.

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