

Mechanisms Mediating the Vasorelaxing Action of Eugenol, a Pungent Oil, on Rabbit Arterial Tissue

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ABSTRACT—The inhibitory actions of eugenol on intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the contractions induced by excess extracellular K^+ concentration ($[\text{K}^+]_o$) in rabbit thoracic aorta were investigated. Application of excess $[\text{K}^+]_o$ solution (30–90 mM) produced contraction and increased the intensity of the Ca^{2+} fluorescence signal. Pretreatment with eugenol (≥ 0.1 mM) reduced both the amplitude of contraction and the intensity of the Ca^{2+} fluorescence signal, but the contraction was more strongly affected than the $[\text{Ca}^{2+}]_i$. Application of eugenol (0.3 mM) to tissue precontracted by 90 mM $[\text{K}^+]_o$ solution (immediately after the removal of the 90 mM $[\text{K}^+]_o$ solution) slowed the decay of the $[\text{Ca}^{2+}]_i$ signal, but it did not change the rate of relaxation. Carbonyl cyanide *m*-chlorophenylhydrozone (10 μM), a mitochondrial metabolic inhibitor, produced a reduction in tension despite a slight increase in $[\text{Ca}^{2+}]_i$ when applied to muscle precontracted by 90 mM $[\text{K}^+]_o$ solution. These results indicate that eugenol relaxes the rabbit thoracic aorta while suppressing the Ca^{2+} -sensitivity and both the uptake and extrusion mechanisms for Ca^{2+} . To judge from the similarities between its actions and those of metabolic inhibitors, eugenol may produce its actions at least partly through metabolic inhibition.

Keywords: Eugenol, Vascular cell, Relaxation, Ca^{2+} , Metabolic inhibition

Eugenol, a natural pungent substance and the chief constituent of clove oil, is commonly used in dental clinics for the sedation of toothache, pulpitis and dental hyperalgesia. Eugenol also produces vasodilation in elastic arteries (1) and increases the blood flow to gingival cutaneous tissue (2). As eugenol and capsaicin have the ability to release substance P and calcitonin-gene related peptide from peripheral nerve endings and dental pulp (2–6), the vasodilating actions of eugenol are thought to be related to those actions on nerve endings (2). This agent may also produce arterial relaxation by a direct action (1). Indeed, in the preliminary experiments, we observed that eugenol inhibited the contractions induced by norepinephrine and excess extracellular K^+ concentration ($[\text{K}^+]_o$) in the endothelium- and adventitia-denuded preparations of rabbit ear artery. Very few papers have been published on the vasodilating actions of eugenol, and we felt that the time had come to elucidate the mechanisms underlying the presumed direct and indirect

actions of this agent on vascular tissues.

To this end, we performed experiments in which we made recordings of isometric tension and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in arterial tissue, while investigating the effects of eugenol on the contractions and changes in $[\text{Ca}^{2+}]_i$ induced by excess $[\text{K}^+]_o$ solution.

MATERIALS AND METHODS

Tissue preparation

Male rabbits (Nippon White, 1.5–1.7 kg) were anesthetized by sodium pentobarbital (40 mg/kg, i.v.) and exsanguinated, permission for the procedures used having been granted by the Animal Research Committee of Fukuoka Dental College. The thoracic aorta was dissected out, and surrounding connective tissues were carefully removed. For the measurement of Ca^{2+} fluorescence, adventitia and intima were carefully removed from the thoracic aorta under a binocular microscope, and small segments (2 × 7 mm) of aorta were prepared. The $[\text{Ca}^{2+}]_i$ was measured using the Ca^{2+} fluorescence indi-

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cator fura-2 (fura-2 AM) (7). Fura-2 AM (Wako Pure Chem., Osaka) was loaded into the cell in the presence of 5% calf serum (pH=7.4) at 20°C overnight, using the method described by Hirano et al. (8) with only a slight modification. Modified Krebs solution (32–35°C) was superfused at a flow rate of 25 ml/min during measurements of Ca^{2+} fluorescence.

Recording of Ca^{2+} fluorescence

Both ends of the preparation were tied with fine threads; one end was fixed to a wire and the other to a force displacement transducer (TB-651T; Nihon Kohden, Tokyo). Two excitation beams (340 and 380 nm) were alternately transmitted (every 1 sec) via small glass fibers ϕ 0.8 mm placed over the preparation, and the emission signal (500 nm) was captured using a glass fiber rod ϕ 4.0 mm placed on the opposite side. The fura-2 signal was recorded by means of a Ca^{2+} spectrometer (FP-777; Japan Spectroscopic, Tokyo) at 340- and 380-nm wavelengths. The results so obtained were stored in a personal computer (Macintosh 7100; Apple Japan, Tokyo) through an A/D converter (MacLab 8s; AD Instruments, Castle Hill, Australia). In the present experiments, we did not calculate absolute values of $[\text{Ca}^{2+}]_i$, but expressed the data as the ratio of the signals obtained at the two wavelengths. In preliminary experiments, we confirmed that eugenol (≤ 1 mM) did not modify the fura-2 fluorescence signals in modified Krebs solution.

Solutions and drugs

The modified Krebs solution used in the present experiments had the following ionic composition: 121.9 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl_2 , 2.5 mM CaCl_2 , 15.5 mM NaHCO_3 , 1.2 mM KH_2PO_4 and 11.5 mM glucose. The pH of the solution was adjusted to 7.3–7.4 using 5% CO_2 –95% O_2 . The drugs used in the present experiments were eugenol, nicardipine and carbonyl cyanide *m*-chlorophenylhydrozone (CCCP) (Sigma Chem., St. Louis, MO, USA). Eugenol was dissolved in dimethyl sulfoxide (DMSO) (Sigma Chem.) and diluted with Krebs solution with the aid of sonication (Branson B2200, Emerson-Japan, Tokyo) just before application. The final concentration of DMSO was kept at less than 0.1%, and this concentration of DMSO was confirmed to have no effect on either the $[\text{Ca}^{2+}]_i$ or tension.

Statistics

The results are expressed as the mean \pm S.D. Statistical significance was assessed using a Student's *t*-test, and *P* values less than 0.05 were considered to be significant.

RESULTS

Simultaneous recordings of contraction and Ca^{2+} fluorescence during application of excess $[\text{K}^+]_o$ solution to segments of the thoracic aorta

Superfusion of excess $[\text{K}^+]_o$ solution (≥ 30 mM) pro-

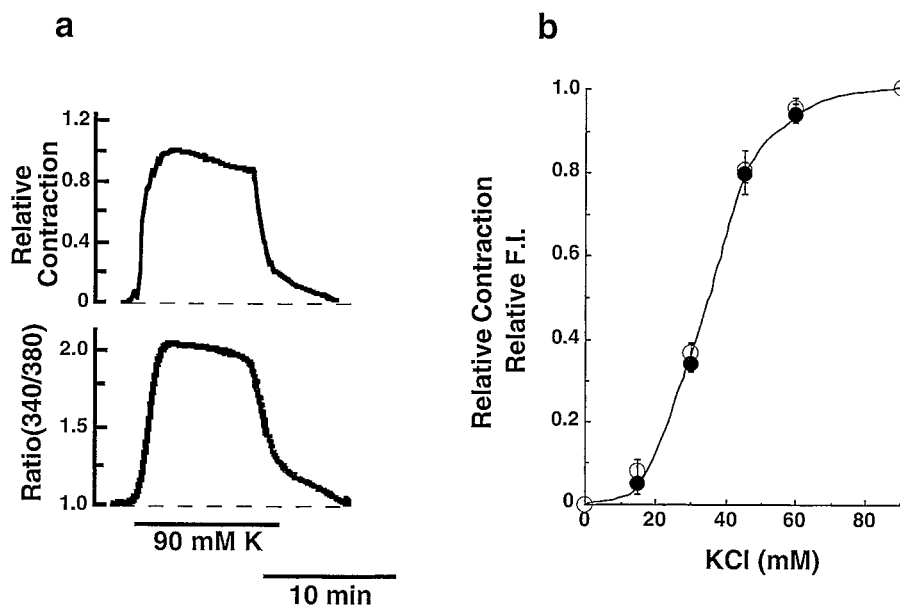


Fig. 1. Simultaneous recordings of the contraction and Ca^{2+} fluorescence signal induced by 90 mM $[\text{K}^+]_o$ solution. a: The peak amplitude of contraction and the basal Ca^{2+} fluorescence signal were each normalized as 1.0. Excess $[\text{K}^+]_o$ solution was applied at the time indicated by the bars. b: Relationships between $[\text{K}^+]_o$ and relative amplitude of contraction (open circles, tension) and Ca^{2+} fluorescence signal (closed circles, F.I.). Responses induced by 90 mM $[\text{K}^+]_o$ solution were normalized as 1.0. Each symbol indicates the mean \pm S.D. of 6–7 observations.

duced a tonic contraction accompanied by a rise in the Ca^{2+} fluorescence signal. Figure 1a shows simultaneous recordings of the contraction and Ca^{2+} signal induced by 90 mM $[\text{K}^+]_o$ solution and shows that after removal of excess $[\text{K}^+]_o$ solution, both contraction and Ca^{2+} signal returned to their control levels. As shown in Fig. 1b, the relationships between $[\text{K}^+]_o$ and the amplitude of contraction (open circles) or the intensity of the Ca^{2+} fluorescence signal (closed circles) were indistinguishable from each other.

Effects of eugenol on the excess $[\text{K}^+]_o$ -induced contractions and Ca^{2+} fluorescence signal

A five-minute pretreatment with 0.1 or 1.0 mM eugenol reduced both the contraction and the Ca^{2+} fluorescence signal induced by 90 mM $[\text{K}^+]_o$ solution (Fig. 2). In the presence of eugenol, the Ca^{2+} fluorescence signals were less sustained (Fig. 2: b and c). On the other hand, the development of contraction was slowed by pretreatment with eugenol. In the example shown in Fig. 2c, 1 mM eugenol reduced the 90 mM $[\text{K}^+]_o$ -induced contraction to 5% of control, while the peak intensity of the Ca^{2+} fluorescence signal was reduced to 30% of control. More than 60-min superfusion with Krebs solution was required to achieve complete restoration of both contraction and Ca^{2+} fluorescence signal after the removal of ≤ 0.3 mM eugenol; however, 1 mM eugenol irreversibly inhibited the 90 mM $[\text{K}^+]_o$ -induced contractions. Figure 3 shows the relationships between the excess $[\text{K}^+]_o$ -induced

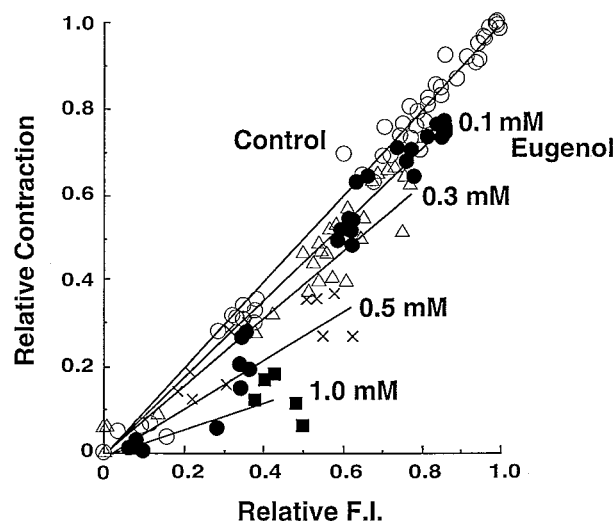


Fig. 3. Relationship between relative amplitude of contraction (tension) and Ca^{2+} fluorescence signal (F.I.) in the presence or absence of eugenol (0.1–1.0 mM). Each data point indicates an individual result obtained at a particular $[\text{K}^+]_o$ concentration (19, 30, 45 or 60 mM). open circles: control, closed circles: 0.1 mM eugenol, triangles: 0.3 mM eugenol, crosses: 0.5 mM eugenol, squares: 1.0 mM eugenol. In all preparations, the 90 mM $[\text{K}^+]_o$ -induced contraction and Ca^{2+} fluorescence signal were recorded and normalized as 1.0.

contraction (30–90 mM) and the peak height of the corresponding Ca^{2+} fluorescence signal in the presence and absence of various concentrations of eugenol. In the absence of eugenol (control), the amplitude of contraction

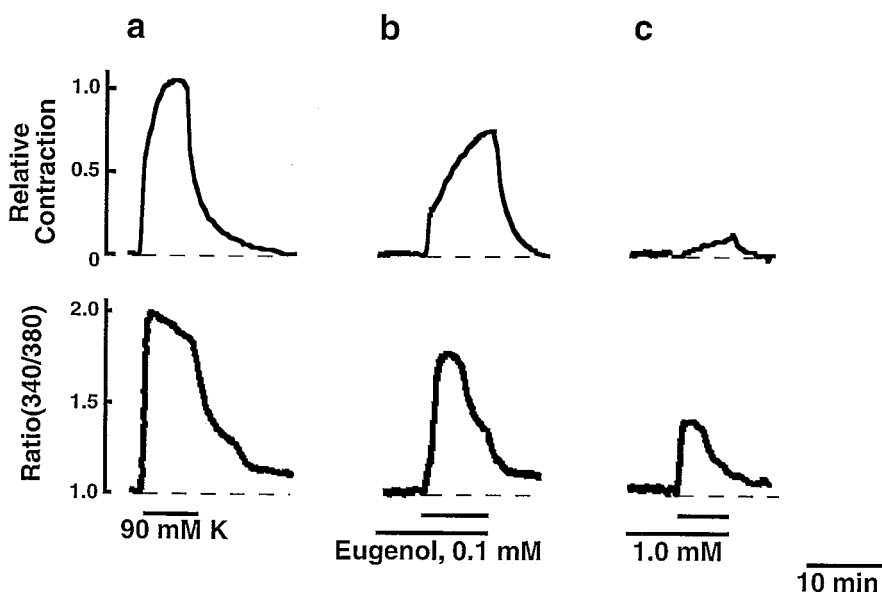


Fig. 2. Effects of eugenol (0.1–1 mM) on the 90 mM $[\text{K}^+]_o$ -induced contraction and Ca^{2+} fluorescence signal. Eugenol was applied 5 min prior to the 90 mM $[\text{K}^+]_o$ solution. a: control, b: 0.1 mM eugenol, c: 1 mM eugenol. a–c were obtained from the same preparation.

increased in proportion to the intensity of the Ca^{2+} fluorescence signal. In the presence of eugenol (0.1–1 mM), the amplitude of contraction was always smaller than that in the control for any given intensity of Ca^{2+} fluorescence signal, and this effect was dose-dependent.

When 0.3 mM eugenol was applied to tissue precontracted by 90 mM $[\text{K}^+]_o$ solution, this agent inhibited the contraction, as it had done in the eugenol-pretreated preparations. On the other hand, this dose of eugenol did not greatly reduce the Ca^{2+} fluorescence signal (Fig. 4: a and b). A selective block of Ca^{2+} entry by nicardipine (1 and 10 μM), a Ca^{2+} antagonist, reduced the contraction and the Ca^{2+} fluorescence signal proportionally (Fig. 4c; 1 μM , 0.21 ± 0.04 of control (tension), 0.25 ± 0.05 of control (Ca^{2+} fluorescence signal); $n=5$). Figure 5 summarizes the effects of various concentrations of eugenol on the 90 mM $[\text{K}^+]_o$ -induced contraction and Ca^{2+} fluorescence signal. As shown in Fig. 5, eugenol inhibited both the contraction and the Ca^{2+} fluorescence signal in a concentration-dependent manner. However, the 50% inhibitory concentrations were different: 0.26 mM for the contraction, but 0.70 mM for $[\text{Ca}^{2+}]_i$. The inhibitory effects of eugenol (≥ 0.1 mM) on the contraction were statistically greater than those on the $[\text{Ca}^{2+}]_i$.

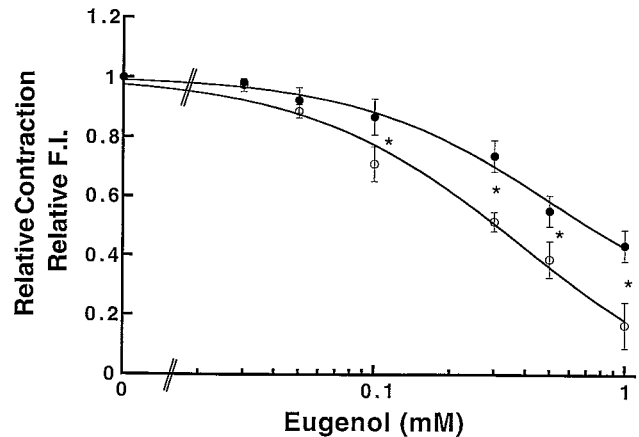


Fig. 5. Relationships between concentration of eugenol and the relative amplitude of contraction and the Ca^{2+} fluorescence signal (F.I.) induced by 90 mM $[\text{K}^+]_o$ solution. Open circles, contraction; closed circles, Ca^{2+} fluorescence signal (F.I.). Responses induced by 90 mM $[\text{K}^+]_o$ in the absence of eugenol were normalized as 1.0. Each symbol indicates the mean \pm S.D. of 6–10 observations. * $P < 0.05$ for the difference between corresponding tension and F.I. data points.

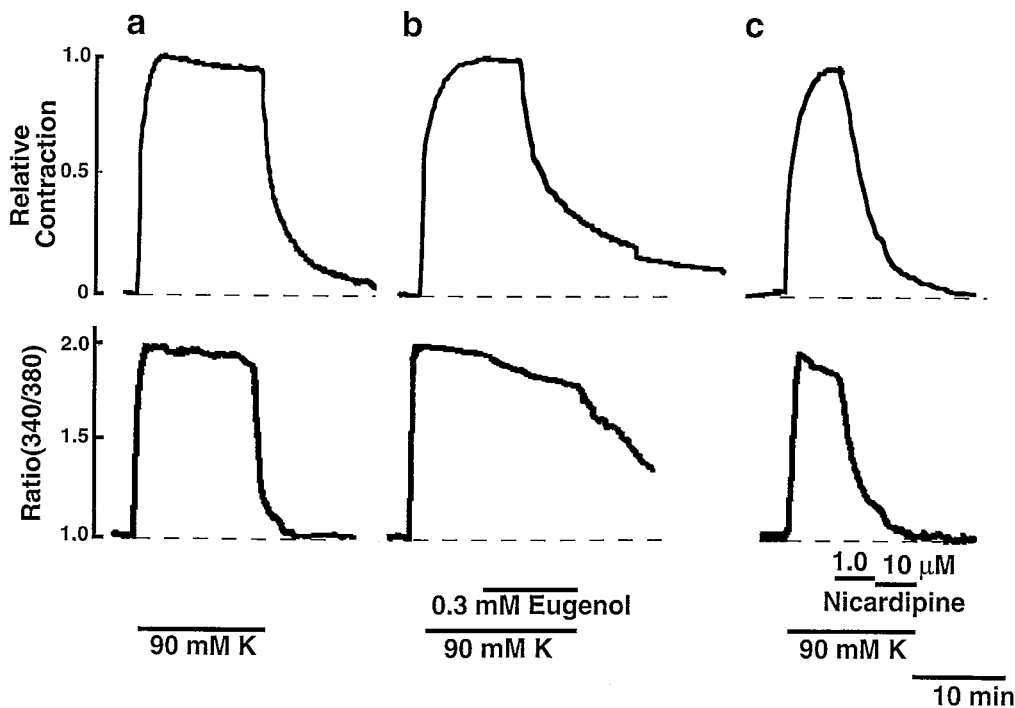


Fig. 4. Effects of eugenol and nicardipine on the contraction and Ca^{2+} fluorescence signal induced by 90 mM $[\text{K}^+]_o$ solution. Eugenol (b) or nicardipine (two different concentrations) (c) were applied at the times indicated by bars after the contraction had reached a sustained level. a: control, b: 0.3 mM eugenol, c: nicardipine (1 and 10 μM). Traces a and b were obtained from the same preparation. Broken line indicates the control level before the application of 90 mM $[\text{K}^+]_o$ solution.

Effects of eugenol on the relaxation time course following the removal of 90 mM $[K^+]_o$ solution

As shown in Fig. 4: a and b, when 0.3 mM eugenol was added to the 90 mM $[K^+]_o$ solution, so that the two were applied to the tissue simultaneously, the rate at which the Ca^{2+} fluorescence signal declined after the removal of the 90 mM $[K^+]_o$ solution was slower than that in the control. To investigate in more detail the effects of eugenol on the rate at which the Ca^{2+} fluorescence signal declined during muscle relaxation, 0.3 mM eugenol was applied to the tissue immediately after the removal of the 90 mM $[K^+]_o$ solution. As shown in Fig. 6a, removal of the 90 mM $[K^+]_o$ solution in the absence of eugenol led to a rapid relaxation of the contraction with a parallel reduction in the Ca^{2+} fluorescence signal. On the other hand, in the presence of 0.3 mM eugenol, the decline in the Ca^{2+} fluorescence signal was much slower, although there was little, if any, change in the time course of the muscle relaxation (Fig. 6b).

Effects of CCCP on the 90 mM $[K^+]_o$ -induced contraction

Recently, Taggart et al. (9) reported that cyanide, a metabolic inhibitor, produced vasorelaxation without reduction of $[Ca^{2+}]_i$ in the rat uterine smooth muscle. In order to compare the actions of eugenol on the 90 mM $[K^+]_o$ -induced contraction to those of metabolic inhibi-

tors, we applied CCCP ($<10 \mu M$), a mitochondrial phosphorylation uncoupler, in aorta. As shown in Fig. 7c, 10 μM CCCP elevated the resting tension with an associated increase in the Ca^{2+} fluorescence signal. The maximum intensity of the Ca^{2+} fluorescence signal induced by 90 mM $[K^+]_o$ solution was also enhanced by CCCP (Fig. 7: b and c). However, the maximum amplitude of the concomitant 90 mM $[K^+]_o$ -induced contraction was reduced. Removal of 90 mM $[K^+]_o$ in the presence of CCCP relaxed the tissue and reduced the Ca^{2+} fluorescence signal, but neither returned to the control level. When 1.0 mM eugenol was applied to the tissue immediately after the removal of the 90 mM $[K^+]_o$ solution, the relaxation was more pronounced than that observed when CCCP alone was present (Fig. 7d), although the Ca^{2+} fluorescence signal stayed at the maximum level reached in the presence of CCCP plus 90 mM $[K^+]_o$ (Fig. 7d).

DISCUSSION

The present experiments have revealed that eugenol inhibits the contraction induced by excess $[K^+]_o$ and reduces the associated Ca^{2+} fluorescence signal. However, the inhibition of the $[K^+]_o$ -induced contraction by eugenol was not in proportion to the reduction in the Ca^{2+} fluorescence signal. From our results, we can infer

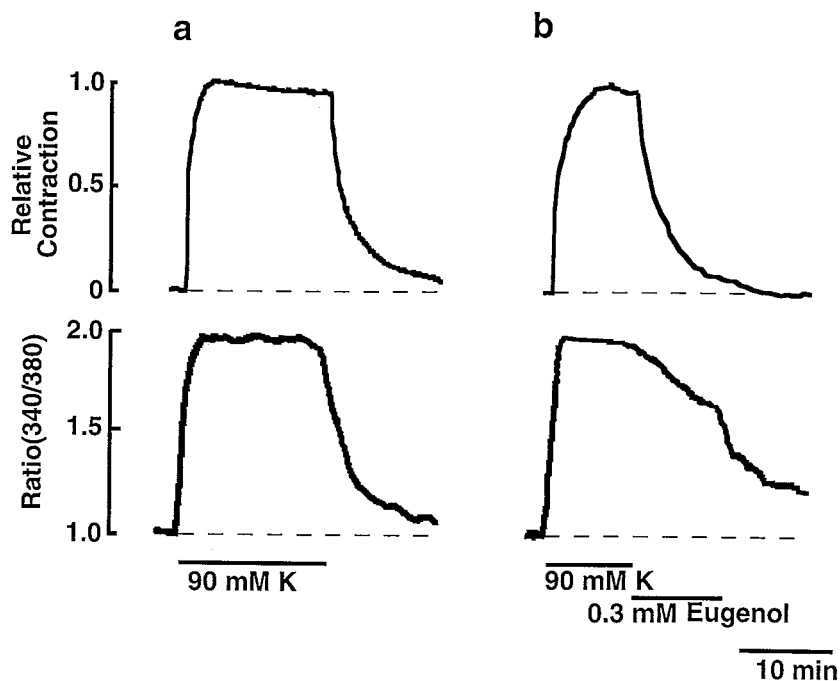


Fig. 6. Effects of 0.3 mM eugenol on the relaxation time-course following the removal of 90 mM $[K^+]_o$ solution. Eugenol was applied at the time indicated by the bar. Traces a and b were obtained from the same preparation. Broken line indicates the control level before application of 90 mM $[K^+]_o$ solution.

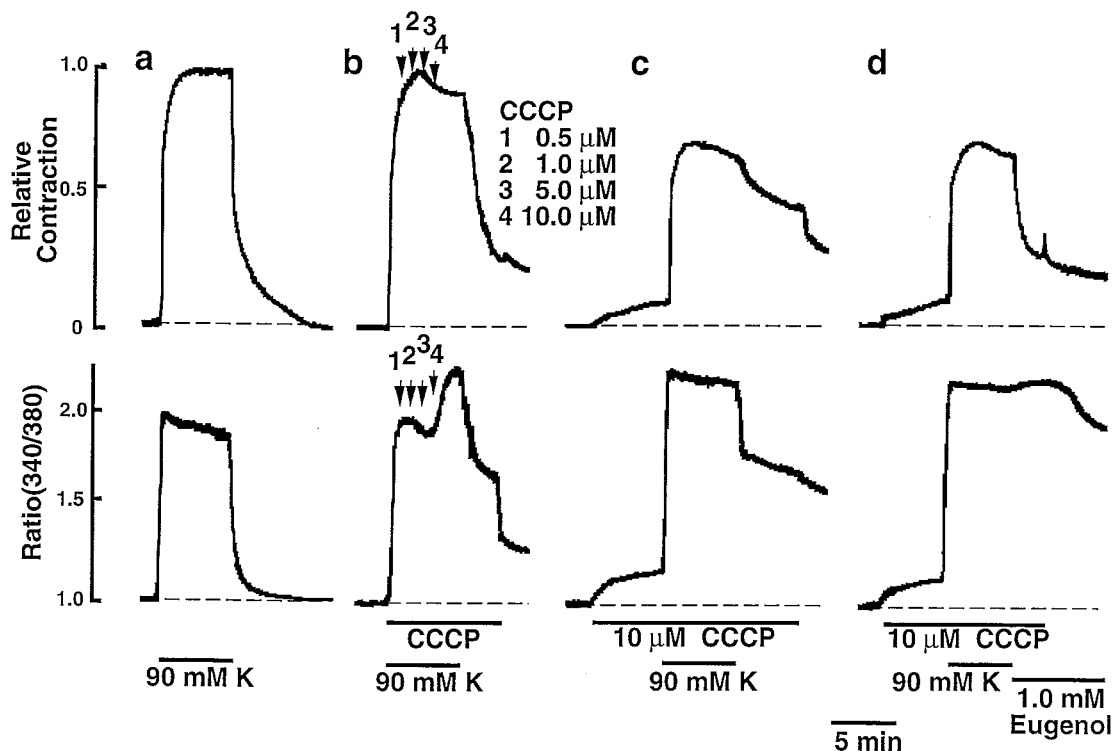


Fig. 7. Effects of CCCP on the resting tension and 90 mM $[K^+]_o$ -induced responses. a: control, b: cumulative application of CCCP (0.5–10 μ M), c: pretreatment with 10 μ M CCCP, d: effect of 1 mM eugenol during muscle relaxation. Broken lines indicate the control levels before application of 90 mM $[K^+]_o$ solution.

that eugenol i) inhibits voltage-dependent Ca^{2+} channels, ii) blocks the Ca^{2+} extrusion mechanisms and iii) inhibits the contractile machinery.

With regard to the mechanisms mediating the vasodilating actions of eugenol, Hume (1) and Leal-Cardoso et al. (10) both concluded that this agent exerts direct inhibitory actions on smooth and skeletal muscle cells. As excess $[K^+]_o$ -induced contractions were mediated by activation of the L-type Ca^{2+} channels, eugenol and other derivatives of hindered phenol were thought to act as Ca^{2+} antagonists (11). As the amplitude of contraction and Ca^{2+} fluorescence signal were proportionally changed by changes in $[K^+]_o$ (Fig. 1), Ca^{2+} antagonists should inhibit the contraction and Ca^{2+} fluorescence signal, proportionally, in the way that nifedipine did. However, we found that inhibition of the $[K^+]_o$ -induced contraction by eugenol was not associated with $[Ca^{2+}]_i$ in the present experiments. This indicated that eugenol did not simply act as a Ca^{2+} antagonist.

On the other hand, as sub-millimolar concentrations of eugenol were found to suppress cellular respiration (12) and to reduce the ATP concentration in the cell (13), it was thought that such toxic actions represent the chief mechanism mediating the vasodilation (1). Metabolic inhibition, caused by hypoxia or application of rotenone

with 2-deoxy glucose, had been reported to inhibit the contractions induced by 40 mM $[K^+]_o$ solution or phenylephrine in smooth muscle preparations (14, 15). Furthermore, Taggart et al. (9) demonstrated that cyanide, an inhibitor of oxidative phosphorylation, produced muscle relaxation without a reduction in $[Ca^{2+}]_i$ in a uterine muscle preparation depolarized by 40 mM $[K^+]_o$ solution. We also confirmed that CCCP (10 μ M) suppressed the 90 mM $[K^+]_o$ -induced contraction, but increased the $[Ca^{2+}]_i$. Because eugenol, cyanide and CCCP reduced the intracellular concentration of ATP (13, 16) and because Ca^{2+} extrusion to the extracellular space and reuptake into the sarcoplasmic reticulum are achieved mainly through activation of Ca^{2+} -dependent ATPase, loss of ATP might conceivably cause a maintenance of $[Ca^{2+}]_i$ during and after the eugenol treatment. As eugenol inhibits cellular respiration at sub-millimolar concentrations (12), reduces the intracellular concentration of ATP (13) and produces the $[Ca^{2+}]_i$ -independent inhibition of the contraction as observed with metabolic inhibitors (the present experiments), it is plausible that inhibition of the cellular respiration is at least partly responsible for the vasorelaxant effect of this agent.

Eugenol alone produced neither contraction nor an in-

creased $[Ca^{2+}]_i$ in the polarized muscle (in Krebs solution) and slightly reduced $[Ca^{2+}]_i$ during the relaxation in the depolarized muscle (in 90 mM $[K^+]_o$ solution) (Figs. 2 and 4). On the other hand, cyanide and CCCP cause an increase in $[Ca^{2+}]_i$ associated with muscle contraction in the polarized muscle and with muscle relaxation in the depolarized muscle (9, the present experiments). Eugenol and several metabolic inhibitors (cyanide, hypoxia and CCCP) cause intracellular acidification (9, 17–20). However, it was reported that intracellular acidification promoted the contractions (21). Therefore, this acidification could not conceivably account for the vasorelaxation induced by eugenol in the polarized muscle. Additional effects other than metabolic inhibition (loss of ATP, cellular acidification, etc.) should account for the actions of eugenol such as inhibition of the voltage-dependent Ca^{2+} channels and contractile machinery. It is worth to note that cyanide and hypoxia have inhibited the voltage-dependent Ca^{2+} channels in rat sensory neurons (22) and guinea pig taenia caeci (23), but these have not altered phosphorylation of the myosin light chain (9, 15). Therefore, eugenol might be expected to inhibit the voltage-dependent Ca^{2+} channels through its actions of metabolic inhibition. Further experiments would be required to clarify the differences of the vasodilating mechanisms between eugenol and other metabolic inhibitors.

Veaco et al. (24) reported that the concentration of eugenol in the dental pulp was of the 10 μ molar order several hours after its topical application. This would seem to suggest that the eugenol concentrations used in the present experiments were somewhat higher than those reached during dental treatment. In the endothelium- and adventitia-intact preparation of rabbit ear artery, eugenol, at the same concentrations used in the present experiments, inhibited the norepinephrine- and excess $[K^+]_o$ -induced contractions to the same extent as observed in the present experiments (1; H. Nishijima and R. Uchida, unpublished observations). This means that differences of the vascular tissues or presence of endothelium and adventitia do not influence the actions of eugenol. As much lower concentrations of eugenol sufficed to produce vasodilation when longer application is used (25), a low sensitivity to this agent in the present experiment might be due to short application of the eugenol treatment.

In conclusion, eugenol has similar properties to metabolic inhibitors such as decrease in pH_i , loss of ATP in the cell, Ca^{2+} -independent relaxation and maintenance of $[Ca^{2+}]_i$ (9, 13, 18, 20, the present experiments). Therefore, we speculate that eugenol inhibits the contraction in rat aorta through several pathways, including the inhibitory actions on the voltage-dependent Ca^{2+} channels, Ca^{2+} extrusion mechanism and others. As some of ac-

tions are also observed in several metabolic inhibitors, effects of eugenol, resulting from its metabolic inhibition, are probably attributable to its pharmacological actions in vitro.

Acknowledgments

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