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MECHANISMS OF CAFFEINE-INDUCED CONTRACTION AND RELAXATION OF RAT AORTIC SMOOTH MUSCLE

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SUMMARY

1. Using front-surface fluorimetry and Fura-2, we determined the effects of caffeine on cytosolic calcium concentration ($[Ca^{2+}]_i$) and on tension of strips of the rat thoracic aorta. We also determined the effects of caffeine on $^{45}Ca^{2+}$ influx into the strips. The objective was to elucidate the mechanism of contraction and relaxation in vascular smooth muscle, as induced by caffeine.

2. In normal physiological salt solution (PSS), caffeine induced a transient tension development, while it induced a biphasic change in $[Ca^{2+}]_i$. The initial transient peak in $[Ca^{2+}]_i$ which coincided with tension development was followed by a sustained increase. Thus, changes in tension did not follow changes in $[Ca^{2+}]_i$. In Ca^{2+}-free PSS, both the caffeine-induced contraction and the increase in $[Ca^{2+}]_i$ were transient. It was suggested that in both the presence and absence of extracellular Ca^{2+}, the transient increase in $[Ca^{2+}]_i$ was due to the release of Ca^{2+} from the intracellular store. Although the sustained increase in $[Ca^{2+}]_i$ depended on extracellular Ca^{2+}, it was not affected by diltiazem, a Ca^{2+} antagonist.

3. Caffeine inhibited the increase in $[Ca^{2+}]_i$ and tension development during 118 mM-K^+ depolarization, in a concentration-dependent manner. The extent of reduction in tension (relaxation) was greater than that expected from the reduction in $[Ca^{2+}]_i$ based on the $[Ca^{2+}]_i$–tension relationship observed with K^+ depolarization. Pretreatment of the strips with ryanodine did not alter the inhibitory effects of caffeine.

4. Caffeine inhibited the increased $[Ca^{2+}]_i$ and developed tension during stimulation by 10^{-5} M-noradrenaline, in a concentration-dependent manner.

5. Dibutyryl cAMP (10^{-4} M) inhibited both high K^+-induced and noradrenaline-induced tension development. Inhibition of an increase in $[Ca^{2+}]_i$ in relation to the inhibition of tension during noradrenaline stimulation was much greater than that in 118 mM-K^+ depolarization.

6. Although caffeine *per se* had no effect on $^{45}Ca^{2+}$ influx in the strips in normal PSS, caffeine did inhibit the increase in $^{45}Ca^{2+}$ influx stimulated by 118 mM-K^+ or by 10^{-5} M-noradrenaline, to a similar extent and with similar IC_{50} values.

7. The characteristic features of the effects of caffeine on vascular smooth muscle,

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i.e. the transient nature of contraction and the relaxation of precontracted strips could be explained as follows: caffeine is able to reduce $[Ca^{2+}]_i$ after releasing Ca^{2+} from intracellular stores; however, this may play a minor role. Independent of the $[Ca^{2+}]_i$ reduction, the second messenger, cAMP, might directly influence the $[Ca^{2+}]_i$–tension relationship, and if so, would play a major role.

INTRODUCTION

Since the discovery of a unique action to release Ca^{2+} directly and specifically from the sarcoplasmic reticulum (SR) (Weber & Herz, 1968), caffeine has been given a special position among drugs. The use of caffeine as a pharmacological tool (Endo, 1977) provided important findings on properties of the SR in smooth muscle (Kuriyama, Itoh, Suzuki, Kitamura & Itoh, 1982; Somlyo, 1985; van Breemen & Saida, 1989). Most of the studies were based on a pharmacological assumption, that is, caffeine-induced tension development reflects the amount of Ca^{2+} released from the SR. However, this assumption may not be appropriate for all preparations because the effect of caffeine on vascular smooth muscle contractility varies considerably among different preparations and this variability is not related to the volume of the SR (Leijten & van Breemen, 1984). For example, it was reported that the caffeine-induced contraction of the rabbit aorta was much smaller than that induced by noradrenaline (NA), though a comparable amount of $^{45}Ca^{2+}$ was released by maximal doses of caffeine and NA (Leijten & van Breemen, 1984). Thus, in such preparations, the questions remained as to why the caffeine-induced tension was so small.

On the other hand, caffeine relaxes various species of smooth muscles, including vascular, bronchial and smooth muscles of the biliary and gastrointestinal tracts (Arnaud, 1987). It was reported that caffeine inhibited high-K^+- and NA-induced contractions in vascular smooth muscles from the rabbit main pulmonary artery (Casteels, Kitamura, Kuriyama & Suzuki, 1977; Ito, Suzuki & Kuriyama, 1977), the rabbit aorta (Leijten & van Breemen, 1984; Ahn, Karaki & Urakawa, 1988) and the rat aorta (Sato, Ozaki & Karaki, 1988). Caffeine was shown to act as a phosphodiesterase inhibitor and to increase the adenosine 3′,5′-cyclic monophosphate content (Butcher & Sutherland, 1962). Cyclic adenosine monophosphate (cAMP) may facilitate Ca^{2+} uptake into the SR (Saida & van Breemen, 1984) or Ca^{2+} extrusion (Suematsu, Hirata & Kuriyama, 1984), and hence may decrease the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). In addition, it was reported that cAMP inhibited myosin light chain kinase activity (Adelstein, Conti, Hathaway & Klee, 1978) and the Ca^{2+}-induced contractions in skinned muscles (Kerrick & Hoar, 1981; Rüegg, Sparrow & Mrwa, 1981). However, a discrepancy was noted between cAMP contents in the presence of caffeine and the potency of the inhibitory effects of caffeine on the contractions (Leijten & van Breemen, 1984). More recently, it was reported that caffeine inhibited $^{45}Ca^{2+}$ influx stimulated by high-K^+ depolarization and by NA (Ahn *et al.* 1988), decreased $[Ca^{2+}]_i$ (Sato *et al.* 1988), and directly inhibited the contractile apparatus (Ozaki, Kasai, Hori, Sato, Ishihara & Karaki, 1990). The relative importance of these mechanisms in the contraction and relaxation of vascular smooth muscle induced by caffeine remains to be elucidated.

In the present study, using front-surface fluorimetry of Fura-2, we recorded

changes in the $[Ca^{2+}]_i$–tension relation induced by caffeine, in a quantitative manner, in intact smooth muscle from the rat aorta. We also measured the effects of caffeine on $^{45}Ca^{2+}$ influx and compared the results with changes in $[Ca^{2+}]_i$. In addition to the effect on $[Ca^{2+}]_i$ homeostasis, the inhibitory effects on the $[Ca^{2+}]_i$–tension relation play a major role in the contraction and relaxation induced by caffeine.

METHODS

Tissue preparation

Adult male rats (WKA, 10–12 weeks, 250–300 g) were anaesthetized by giving an intraperitoneal injection of 2 ml kg^{-1} pentobarbitone, and were exsanguinated from the femoral artery. The thoracic aortas were isolated and cut longitudinally. The luminal surface was rubbed gently with a cotton swab to remove the endothelium. The preparations were cut into circular strips ($1 \times 5 \times 0.1$ mm).

Fura-2 loading

The strips were incubated in normal physiological salt solution (PSS) containing 25 μM Fura-2 AM, 0·08 % cremophore and 1 mM-probenecid for 3 h at 37 °C in a dark room. The solutions were bubbled with 95% O_2 and 5% CO_2 throughout the experiments. After loading with Fura-2, the strips were rinsed in normal PSS containing 1 mM-probenecid for 30 min to remove dye in the extracellular space. Emission and excitation spectra for the Fura-2-loaded strips were examined using a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan). The Fura-2-loaded strips showed a fluorescence excitation spectrum with a peak at 340 nm and fluorescence emission spectra with a peak at 500 nm, both at 340 nm and at 380 nm excitation. These characteristics indicated that fluorescence spectra obtained with Fura-2-loaded strips were specific for this dye (Grynkiewicz, Poenie & Tsien, 1985). We added 1 mM-probenecid to all the solutions to prevent intracellular sequestration and secretion of Fura-2 (DiVirgilio, Silversteine & Steinberg, 1988). As shown in Fig. 1, loading of Fura-2 in the presence of 1 mM-probenecid did not affect the time course and the maximum tension induced by either 118 mM-K^+ depolarization or 10^{-6} M-noradrenaline (NA). Application of 10^{-9}–10^{-5} M-acetylcholine during NA-induced contraction reduced neither the fluorescence ratio nor the tension, hence the endothelial cells had been removed (Fig. 1).

Measurement of tension

The Fura-2-loaded strips were mounted vertically in a quartz organ bath. Tension development was measured using a strain gauge (TB-612T, Nihon Koden, Japan). The strips were stimulated by 118 mM-K^+ depolarization five or six times repeatedly before each experiment, and the resting tension was adjusted to 200–300 mg. The developed tension was expressed as a percentage of a control 118 mM-K^+-induced tension development measured at 10 min after application.

Measurement of $[Ca^{2+}]_i$

Changes in the fluorescence intensities of the Fura-2–Ca^{2+} complex in the strips were monitored with a spectrofluorimeter (model CAM-OF-1), specially designed for front-surface fluorimetry of Fura-2, with the collaboration of Japan Spectroscopic Co., Tokyo, Japan (Hirano, Kanaide & Nakamura, 1990; Abe, Kanaide & Nakamura, 1990). In brief, the strips were illuminated by alternating (400 Hz) 340 nm and 380 nm excitation light from a xenon lamp through quartz optic fibres arranged in a concentric inner circle (diameter, 3 mm). Surface fluorescence of the strips was collected by glass optic fibres arranged in an outer circle (diameter, 7 mm) and introduced through a 500 nm bandpass filter into a photomultiplier. Ratio of the fluorescence intensities at 340 nm excitation to those at 380 nm excitation was monitored and expressed as a percentage, assuming that the steady-state values in normal PSS and in 118 mM-K^+ PSS at 10 min after application were 0 and 100%, respectively. The absolute value of $[Ca^{2+}]_i$ was calculated from the fluorescence ratio (R) using the following equation (Grynkiewicz *et al.* 1985)

$$[Ca^{2+}]_i = K_d (R - R_{min})/(R_{max} - R),$$

where K_d is a dissociation constant for Fura-2 (224 nM, Grynkiewicz *et al.* 1985). R_{max} and R_{min} were determined by the addition of 5 μM-ionomycin in normal PSS, and in Ca^{2+}-free PSS containing 2 mM-EGTA, respectively. The absolute values of $[Ca^{2+}]_i$ (means $\pm$ S.D., $n = 10$) at rest (0%) and during 118 mM-K^+ depolarization (100%) were 207.8 ± 35.7 nM and 639.6 ± 85.8 nM, respectively.

Measurement of $^{45}Ca^{2+}$ influx

Calcium-45 influx experiments were performed according to the method of van Breemen, Hwang & Meisheri (1981). In brief, ring preparations of the rat aorta (length about 5 mm) were incubated for 2 min in solutions containing $^{45}Ca^{2+}$ (4 μCi ml^{-1}) bubbled with 95% O_2 and 5% CO_2 at 37 °C. Extracellular $^{45}Ca^{2+}$ was washed out in ice-cold Ca^{2+}-free PSS containing 2 mM-EGTA for 45 min. The sample were weighed and left overnight in a vial containing 1 ml of Ca^{2+}-free PSS, at room temperature. After the addition of a 10 ml liquid scintillator cocktail (ACS II, Amersham Co., USA), the radioactivity was counted using a liquid scintillation counter (LCS-3500, Aloka Co, Tokyo, Japan). The amount of Ca^{2+} estimated from incorporation of $^{45}Ca^{2+}$ into the samples was expressed as micromoles per kilogram per two minutes.

Drugs and solutions

Caffeine, noradrenaline, ryanodine and Fura-2 were purchased from Katayama Chemicals (Osaka, Japan), Sigma Chemical Co. (St Louis, MO, USA), Research Biochemicals Inc. (Natick, MA, USA), and Molecular Probes Inc. (Eugene, OR, USA), respectively. $^{45}Ca^{2+}$ (1·26 GBq mg^{-1}) was from DuPont/NEN (Billerica, MA, USA). Compositions of the solutions were as follows (in mM). Normal PSS: NaCl, 123, KCl, 4·7; $NaHCO_3$, 15·5; KH_2PO_4, 1·2; $MgCl_2$, 1·2; $CaCl_2$, 1·25; and D-glucose, 11·5 (pH 7·4). The composition of 118 mM-K^+ PSS was the same as normal PSS, except for the equimolar substitution of KCl for NaCl. Ca^{2+}-free PSS contained 2 mM-EGTA. A stock solution of probenecid was made in 0·1 M-NaOH solution and the pH of the solution was adjusted to 7·4 with HCl.

Statistical analysis

Results were expressed as means $\pm$ standard deviation. We used one-way analysis of variance to test dose dependence and Student's t test or Cochlan Cox's test for the analysis. P values less than 0·05 were considered to have a statistical significance. We obtained the EC_{50} or IC_{50} value for each dose–response curve, using the four-parameter logistic model (De Lean, Munson & Rodbard, 1978), except for Figs 4*A*, 6*B*, 6*C* and 8*B*, where the data did not fit the model and the IC_{50} was determined graphically. $[Ca^{2+}]_i$–tension curves for contractions induced by the cumulative addition of extracellular Ca^{2+} during 118 mM-K^+ depolarization were obtained by fitting the data to Hill's equation (Segel, 1976):

$$\log[T/(T_{max}-T)] = \alpha(\log[Ca^{2+}]_i - \log K_d), \quad (1)$$

where T represents measured value of tension development (%). α is the Hill coefficient. K_d is an apparent dissociation constant and equal to a $[Ca^{2+}]_i$ giving half the maximal tension. T_{max} is the maximal tension estimated by using the following equation and least-squares method (Scatchard, 1949):

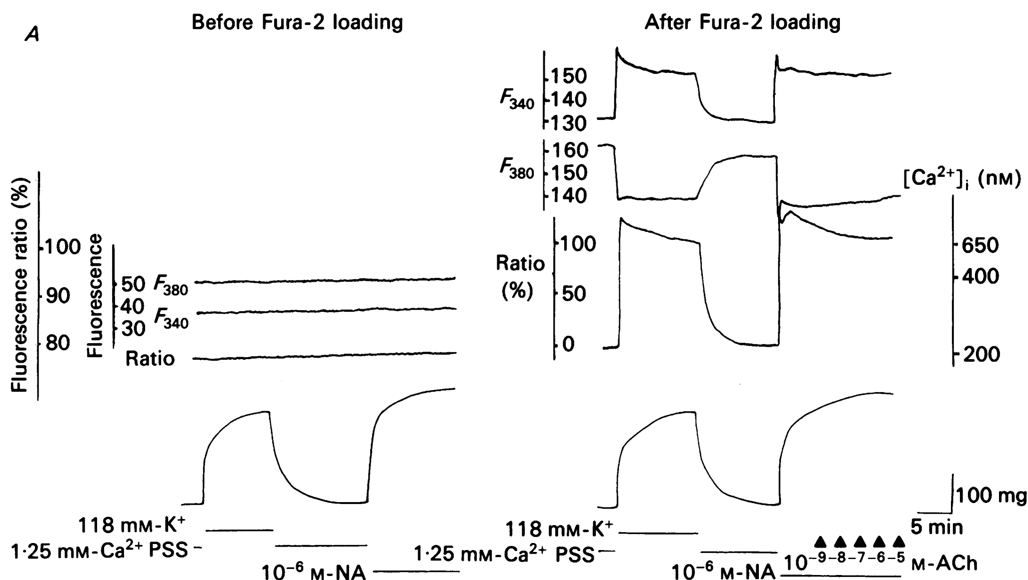
$$T/[Ca^{2+}]_o = (T_{max}-T)/K', \quad (2)$$

where $[Ca^{2+}]_o$ represents extracellular Ca^{2+} concentration and K' is a constant.

RESULTS

$[Ca^{2+}]_i$–tension relationship induced by K^+ depolarization and noradrenaline

As shown in Fig. 1*A*, when vascular strips were exposed to 118 mM-K^+, the fluorescence ratio and tension reached maximum levels within 30 s and 10 min, respectively. These levels were either sustained or were slightly reduced during depolarization and depended on the concentration of K^+. When 10^{-5} M-NA was added to normal PSS, the fluorescence ratio rose abruptly and reached the first peak within a few seconds. After a slight dip at 15 s, it reached a second peak at 40 $\pm$ 8 s ($n = 16$), then gradually declined to reach a sustained level within 10 min. The tension developed rapidly and reached a plateau within 10 min. The level of fluorescence ratio and the extent of tension development were concentration dependent.



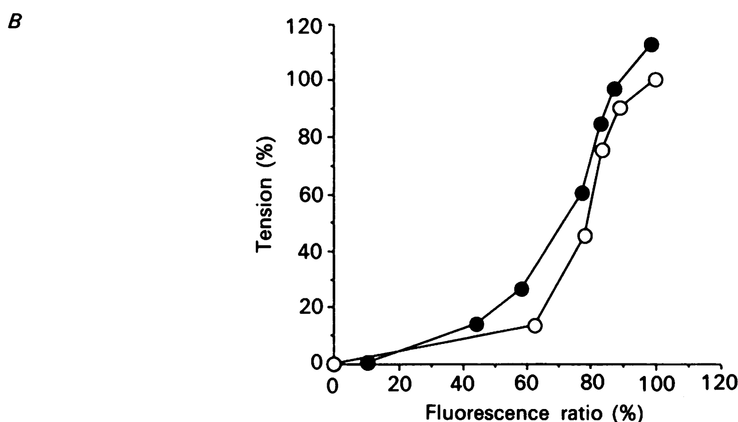


Fig. 1. Changes in fluorescence and tension induced by depolarization with 118 mM-K^+ PSS and by 10^{-6} M-NA in rat aortic strips. *A*, effects of Fura-2 loading in the presence of probenecid. The first and the second traces from the top show the fluorescence intensities (measured in arbitrary units) at 500 nm emission obtained at 340 and 380 nm excitations. The third trace shows the ratio of fluorescence intensities at 340 nm excitation to that at 380 nm excitation. The bottom trace shows tension. Before Fura-2 loading, there were no changes in the fluorescence intensities at either 340 or 380 nm excitations, or in the ratio of these fluorescence intensities during depolarization and 10^{-6} M-NA stimulation. After Fura-2 loading, the fluorescence intensities of resting muscle increased by about 3 times. The fluorescence ratio was increased by depolarization with 118 mM-K^+ PSS and NA stimulation. Fura-2 loading in the presence of probenecid did not affect the contractions elicited by 118 mM-K^+ depolarization (234 ± 10·2 and 236 ± 11·6 mg) or NA (280 ± 9·5 and 284 ± 8·8 mg). Application of 10^{-9}–10^{-5} M-acetylcholine (ACh) during the NA-induced contraction did not change the fluorescence ratio and the tension. ▲ indicate the times when appropriate amounts of stock solutions of drugs were added to the bath. *B*, $[Ca^{2+}]_i$–tension relationships obtained at sustained or plateau levels induced by various concentrations of K^+ (○, 20–118 mM) and NA (●, 10^{-9}–10^{-5} M).

$[Ca^{2+}]_i$–tension relationships obtained at sustained or plateau levels induced by various concentrations of K^+ (20–118 mM) and NA (10^{-9}–10^{-5} M) are shown in Fig. 1*B*. NA caused a greater tension development for a given change in $[Ca^{2+}]_i$ than did K^+, thus, the $[Ca^{2+}]_i$–tension curve was shifted to the left.

Effects of caffeine on cytosolic Ca^{2+} concentration and on tension

Representative time courses of changes in Fura-2 fluorescence ratio and tension evoked by 10 mM-caffeine in normal PSS and in Ca^{2+}-free PSS are shown in Fig. 2*A* and *B*, respectively. Prior to the application of caffeine, 118 mM-K^+ PSS was applied to determine the 100% level of fluorescence ratio and tension in the presence of 1·25 mM-Ca^{2+}. In normal PSS, subsequent application of 10 mM-caffeine induced a rapid increase in fluorescence ratio and tension. The fluorescence ratio reached a peak ($169·6 \pm 28·2$%, $n = 4$) at 10–15 s after the application of caffeine (phasic component), and then rapidly declined to a steady level ($76·6 \pm 8·7$% at 5 min, $n = 4$), which was much higher than the resting level (0%) and was sustained for at least 30 min (sustained component). When caffeine was removed, the fluorescence ratio rapidly declined to reach below the resting level, then, gradually returned to the resting level. At 15–20 s after beginning the application of caffeine, the tension reached a peak level ($52·4 \pm 11·6$%). Tension development in relation to the level of $[Ca^{2+}]_i$ at this peak (169·6%, Fig. 2) was much smaller than those induced by K^+ depolarization and NA (Fig. 1*B*). The tension returned to below the resting level despite the sustained increase in fluorescence ratio. Thus, there was a marked dissociation between the changes in fluorescence ratio and the changes in tension induced by 10 mM-caffeine. After re-loading the intracellular Ca^{2+} store sites by 118 mM-K^+ depolarization, the strips were treated with diltiazem (Fig. 2*A*). The addition of 10^{-5} M-diltiazem slightly decreased the fluorescence ratio to below the resting level. At 15 min incubation with diltiazem, the strip was stimulated by 10 mM-caffeine. The phasic components of changes in the fluorescence ratio and tension, if any, were slightly depressed. The second, sustained, component of changes in the fluorescence ratio was not affected. The peak levels of phasic components of changes in fluorescence ratio and tension induced by caffeine in normal PSS were concentration dependent in a range of 0·5–50 mM (Fig. 2*C* and *D*). At concentrations over 20 mM, while the peak fluorescence ratio was higher than 200%, the peak tension was much lower than 100%. EC_{50} values for the peak fluorescence ratio and the peak tension were $4·9 \pm 0·9$ mM and $5·5 \pm 0·9$ mM, respectively. A tonic component of the increase in fluorescence ratio was observed at concentrations over 2 mM with the maximal response seen at 10 mM (Fig. 2*C*). Caffeine did not induce sustained tension in normal PSS, at the concentrations tested. Thus, for a given change in $[Ca^{2+}]_i$, caffeine caused a smaller tension development than did K^+ depolarization both at the peak of the phasic component and during the sustained component: $[Ca^{2+}]_i$–tension relationships both at the peak of the phasic component and at the sustained component of caffeine-induced contraction were shifted to the right compared with that of K^+ depolarization (Fig. 2*E*).

Figure 2*B* shows representative time courses of fluorescence ratio and tension development induced by 10 mM-caffeine in Ca^{2+}-free PSS containing 2 mM-EGTA. Following recordings of the 100% level of responses of the fluorescence ratio and

tension in 1·25 mM-Ca^{2+} PSS, a strip was exposed to Ca^{2+}-free PSS. The level of fluorescence ratio gradually decreased to reach $-64{\cdot}1 \pm 5{\cdot}0$ % at 10 min ([Ca^{2+}]$_i$, from 208·6 nM to 104·2 nM) with no change in the tension.

In the absence of extracellular Ca^{2+}, application of 10 mM-caffeine induced transient increases in both the fluorescence ratio and tension. The changes in fluorescence ratio and tension were concentration dependent in a range of 5–50 mM (Fig. 2*C* and *D*). EC_{50} values could not be determined, because the data did not give a definite maximal response.

Inhibitory effects of caffeine on 118 mM-K^+ contractions

Since it became apparent that tension development related to the increase in [Ca^{2+}]$_i$ was markedly depressed during contraction induced by caffeine, further studies were carried out to explore the mechanisms of these inhibitory effects of caffeine on smooth muscle contraction. At the steady state (8–10 min) of 118 mM-K^+-induced contraction, application of caffeine (5 or 50 mM) caused an initial transient increase, to a sharp peak, followed by a decrease in both fluorescence ratio and tension (Fig. 3*A* and *B*). As shown in Fig. 3*A*, 5 mM-caffeine decreased the tension almost to the resting level ($4{\cdot}6 \pm 2{\cdot}6$ %, $n = 4$, measured at 10 min after the application of caffeine), while the fluorescence ratio was decreased but still remained at a relatively higher level than the resting level ($68{\cdot}0 \pm 4{\cdot}1$ %, [Ca^{2+}]$_i$ = 428·7 nM, $n = 4$). As shown in Fig. 3*B*, 50 mM-caffeine decreased the fluorescence ratio to the resting level ($-2{\cdot}6 \pm 7{\cdot}6$ %, [Ca^{2+}]$_i$ = 203·0 nM, $n = 4$) and the tension below the resting level ($-13{\cdot}9 \pm 4{\cdot}4$ %). Figure 4*A* and *B* show the concentration–response relationships of the inhibitory effects of caffeine on the 118 mM-K^+-induced increases in fluorescence ratio and tension, respectively. The inhibitory effects of caffeine were concentration dependent in a range of 0·5–50 mM (Fig. 4). IC_{50} for the inhibitory effects of caffeine on the fluorescence ratio (about 10 mM) was higher than that for the tension ($2{\cdot}1 \pm 0{\cdot}04$ mM, $n = 4$).

Contribution of uptake of Ca^{2+} into intracellular store sites to the decrease in [Ca^{2+}]$_i$ induced by caffeine was investigated using ryanodine, which is known to lock Ca^{2+} channels of the store sites at the open state and functionally deplete Ca^{2+} in the stores (Stuko & Kenyon, 1983). In a strip pretreated with ryanodine (10^{-5} M) and caffeine (20 mM) according to the protocol of Sato *et al.* (1988), the extent and time course of changes in the fluorescence ratio and tension induced by 118 mM-K^+ were similar to those without ryanodine pretreatment. When caffeine was added during the 118 mM-K^+-induced contraction after pretreatment with ryanodine (10^{-5} M) and caffeine (20 mM), changes in fluorescence ratio and tension were essentially similar to those seen without pretreatment, except that there were no transient, sharp elevations in fluorescence ratio and tension (data not shown). The inhibitory effects of caffeine on 118 mM-K^+-induced changes in fluorescence ratio and tension in the strip pretreated with ryanodine were also concentration dependent (Fig. 4*A* and *B*).

Effects of caffeine on the [Ca^{2+}]$_i$–tension relationship obtained by re-admitting Ca^{2+} during high K^+ depolarization

To further investigate the inhibitory actions of caffeine on the contraction induced

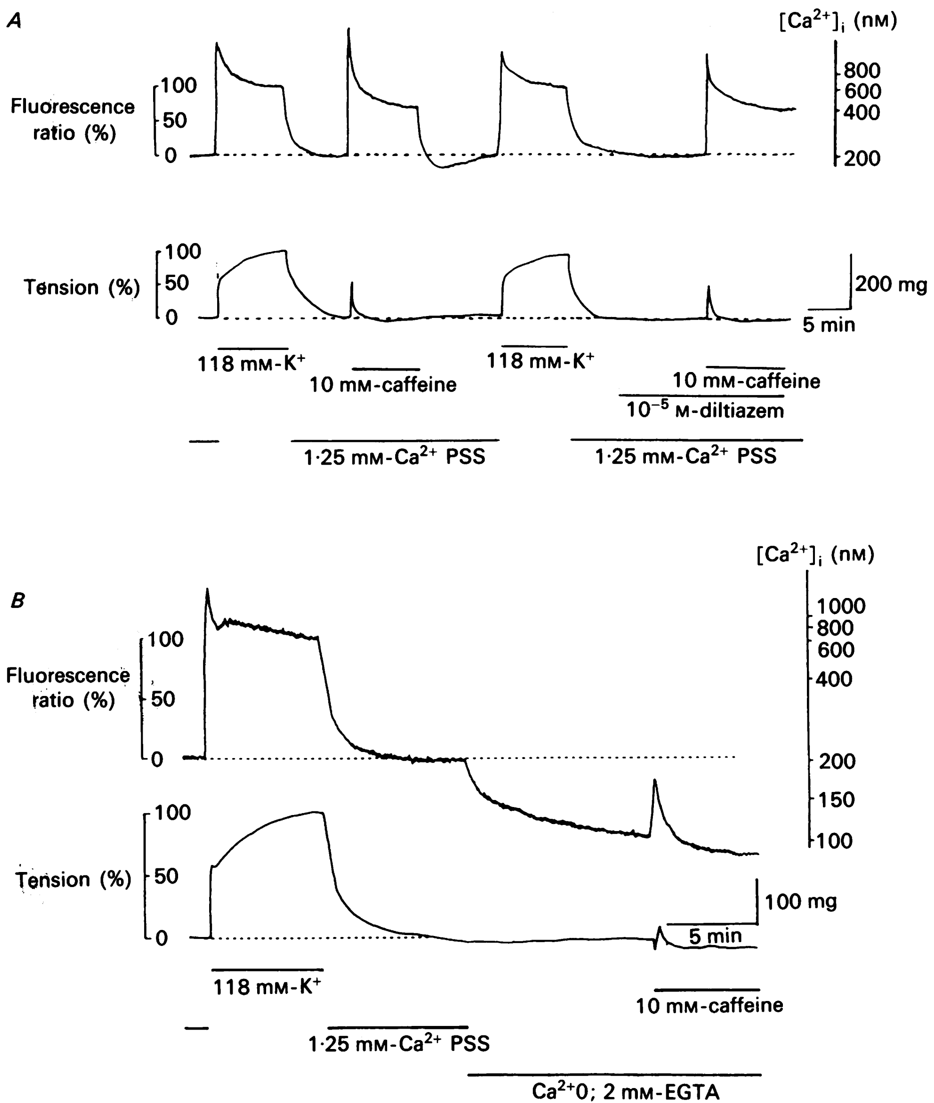
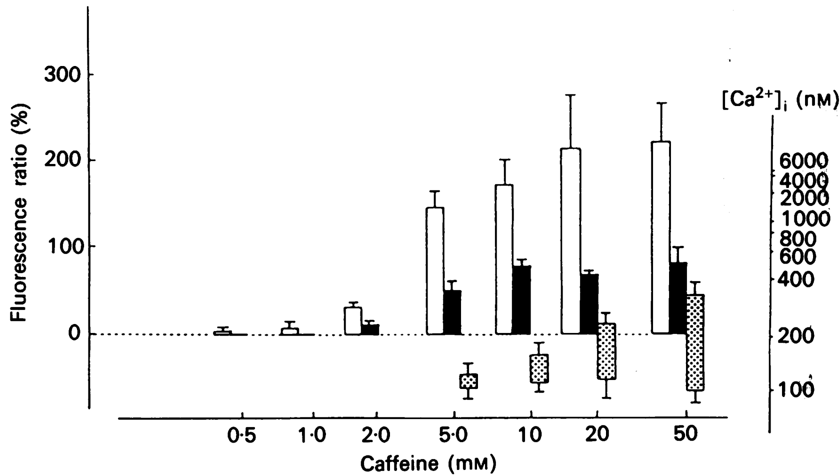
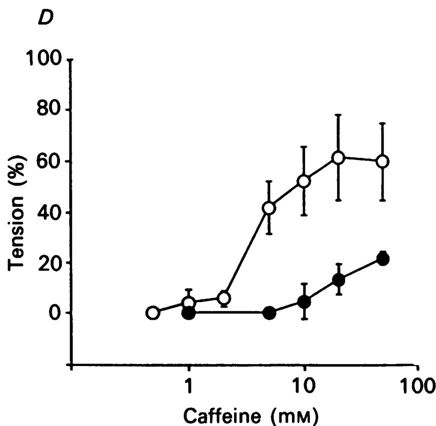


Fig. 2. For legend see facing page.

by depolarization with high external K^+ PSS, we used the following protocol which facilitated examination of $[Ca^{2+}]_i$–tension relationships in greater ranges of changes in $[Ca^{2+}]_i$ (Hirano, Kanaide & Nakamura, 1989). A strip was exposed to Ca^{2+}-free solution containing 2 mM-EGTA for 10 min, then was depolarized with Ca^{2+}-free (no EGTA) 118 mM-K^+ solution. Successive cumulative additions of $CaCl_2$ to the bath (up to 5 mM) caused stepwise increases in fluorescence ratio and tension (Fig. 5*A*). Calcium chloride at a concentration higher than 5 mM in PSS resulted in precipitation. At an extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) of 5 mM, the fluorescence ratio and the tension were $124·8 \pm 8·2$% ($[Ca^{2+}]_i$ = 912 nM) and $136·6 \pm 7·6$% of the control responses in 118 mM-K^+ PSS containing 1·25 mM-Ca^{2+}, respectively ($n = 4$) (Fig. 5*B*





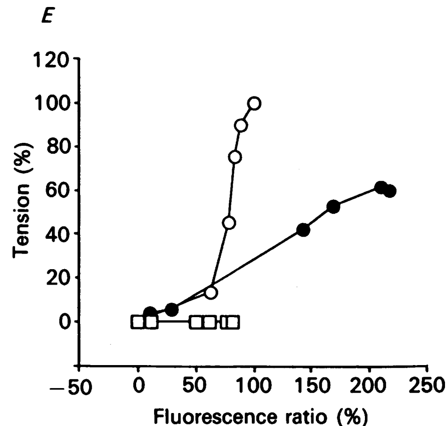


Fig. 2. Changes in fluorescence ratio and tension development induced by caffeine in the presence and the absence of extracellular Ca^{2+}. Representative recording in the presence (*A*) and absence (*B*) of extracellular Ca^{2+}. The upper trace shows fluorescence ratio (340/380 nm excitation) and the bottom trace shows developed tension. *A*, caffeine (10 mM) induced an increase in the fluorescence ratio with phasic and sustained components and a transient contraction in normal PSS. After reloading the Ca^{2+} stores in 118 mM-K^+ PSS, the second application of caffeine induced much the same increase in the fluorescence ratio in the sustained component in the presence of 10^{-5} M-diltiazem. Tension induced by 118 mM-K^+ was $225 \pm 12{\cdot}2$ mg. *B*, caffeine (10 mM) was applied after 10 min incubation in Ca^{2+}-free PSS. The absolute value of tension induced by 118 mM-K^+ was $285 \pm 7{\cdot}8$ mg. *C*, concentration-dependent effects of caffeine on the fluorescence ratio in normal PSS and in Ca^{2+}-free PSS. Open bars and filled bars represent changes measured at each peak of the phasic component ($EC_{50} = 4{\cdot}9 \pm 0{\cdot}9$ mM) and at 5 min after application of caffeine, the sustained component, in normal PSS, respectively. Dotted bars represent the changes in the fluorescence ratio in Ca^{2+}-free PSS. The top and the bottom of each dotted bar represent a peak value and a value just before application of caffeine, respectively. *D*, concentration–response curves of caffeine-induced tension development in normal PSS. (○, $EC_{50} = 5{\cdot}5 \pm 0{\cdot}9$ mM) and in Ca^{2+}-free PSS (●). Data are means ± S.D. from four different experiments. *E*, $[Ca^{2+}]_i$–tension relationships obtained at the peak of the phasic component (●) and at the sustained component (□) of the contraction induced by various concentrations of caffeine. ○, K^+ depolarization (same as Fig. 1*B*).

and C). The $[Ca^{2+}]_i$–tension relationship for this contraction is shown in Fig. 5D. The minimum $[Ca^{2+}]_i$ which produced a detectable contraction was about 200 nM. The tension increased along with an increase in $[Ca^{2+}]_i$ but did not reach a plateau level at 912 nM-$[Ca^{2+}]_i$ ($n = 4$) or at 5 mM-$[Ca^{2+}]_o$. These characteristics were similar to those

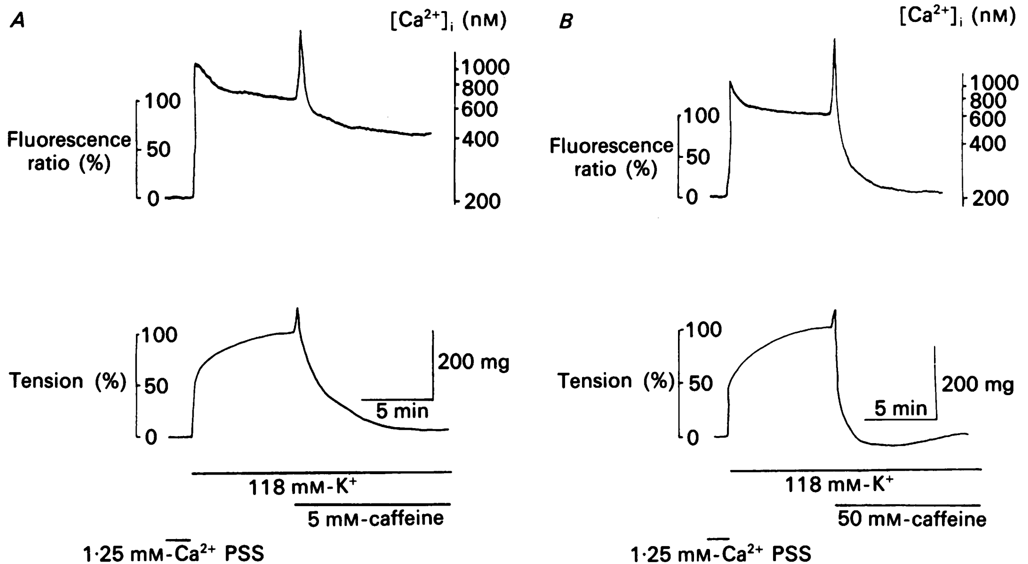


Fig. 3. Effects of caffeine on 118 mM-K^+-induced contraction. A, application of 5 mM-caffeine during the contraction elicited by 118 mM-K^+ depolarization (294 ± 14·2 mg). B, application of 50 mM-caffeine during the 118 mM-K^+-induced contraction (286 ± 8·2 mg).

of Ca^{2+} contractions in skinned fibres (Saida & Nonomura, 1978; Itoh, Kuriyama & Suzuki, 1981). Thus, the extent of an increase in tension induced in intact muscular strips seemed to depend exclusively on changes in Ca^{2+}.

In the presence of 5 mM-caffeine, the extent of an increase in $[Ca^{2+}]_i$ or tension produced by a given $[Ca^{2+}]_o$ was significantly smaller than that in the absence of caffeine (Fig. 5B and C). For example, at 5 mM-$[Ca^{2+}]_o$, the fluorescence ratio and the tension were 110·5 ± 13·3 % ($[Ca^{2+}]_i$ = 733 nM, $n = 4$) and 40·2 ± 3·8 %. In the presence of 10 mM-caffeine, no contraction was observed even though the fluorescence ratio was elevated to 88·9 ± 3·8 % ($[Ca^{2+}]_i$ = 550 nM, $n = 4$), a level of $[Ca^{2+}]_i$ that would induce a contraction equivalent to 90 % of the control 118 mM-K^+ contraction in the absence of caffeine (Fig. 5B and C). Figure 5D shows $[Ca^{2+}]_i$–tension relationships for the 'Ca^{2+}-induced contractions' in the absence and the presence of 2, 5 and 10 mM-caffeine. Since we could not increase $[Ca^{2+}]_i$ over 912 nM because of precipitation, we could not record the maximal tension development (T_{max}). However, in Fig. 5C, the $[Ca^{2+}]_o$–tension curves appeared to resemble hyperbolae. Thus, we fitted the data of Fig. 5C to the Michaelis–Menten equation (equivalent to eqn (2) in Methods) and calculated each T_{max} value using the least-squares method. We fitted the data to Hill's equation (eqn (1)), calculated K_d and the Hill coefficient and constructed $[Ca^{2+}]_i$–tension curves. Values of parameters for each curve are

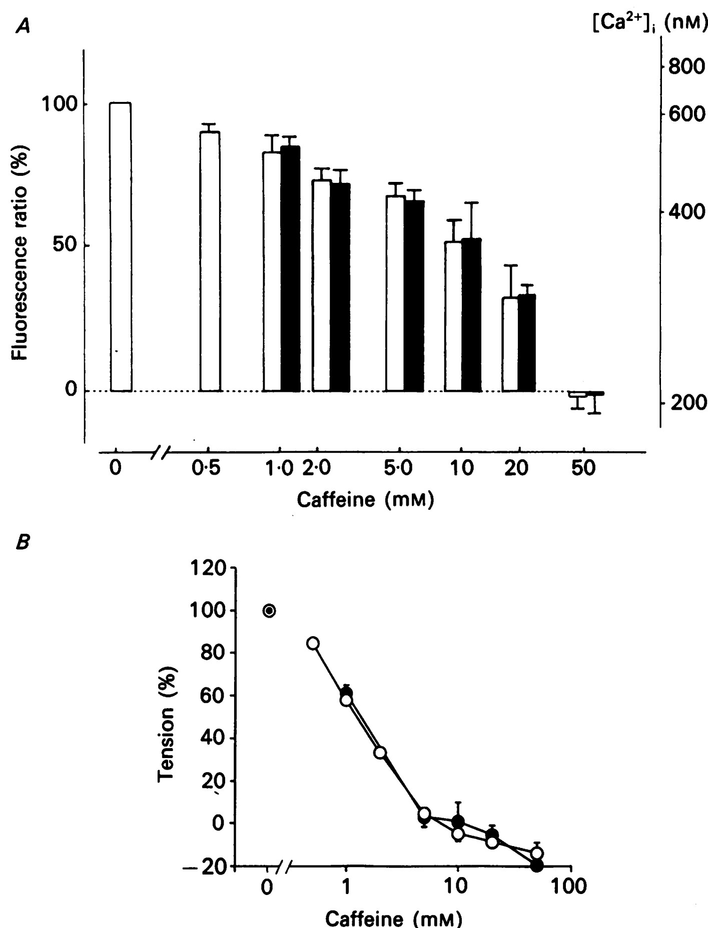


Fig. 4. Concentration-dependent inhibitory effects of caffeine on fluorescence ratio (*A*) and on tension (*B*) during the contraction elicited by 118 mM-K^+ depolarization. The data were obtained at 10 min after exposure to caffeine. *A*, open bars represent percentage fluorescence ratio in the presence of various concentrations of caffeine (IC_{50} of about 10 mM). Filled bars are the result of similar experiments in strips pretreated with 10^{-5} M-ryanodine and 20 mM-caffeine. *B*, ○ represent percentage tension in the presence of various concentrations of caffeine ($IC_{50} = 2·1 \pm 0·04$ mM). ● represent percentage tension in the strips pretreated with 10^{-5} M-ryanodine and 20 mM-caffeine. Data are means ± S.D. from four different experiments.

presented in Table 1. Caffeine decreased the T_{max} and increased the K_d in a dose-dependent manner. The Hill coefficient (α) was not altered in the presence of caffeine.

Inhibitory effects of caffeine on the noradrenaline-induced contraction

At the steady state of contraction induced by 10^{-5} M-noradrenaline (NA) in normal PSS, the fluorescence ratio was $98·5 \pm 4·0$ % and tension was $108·6 \pm 5·4$ % ($n = 4$). As shown in Fig. 6*A*, application of 5 mM-caffeine at 10 min after the application of NA did not induce transient, phasic increases (in contrast to the case of 118 mM-K^+ depolarization, Fig. 3*A* and *B*) but did induce decreases in fluorescence ratio and

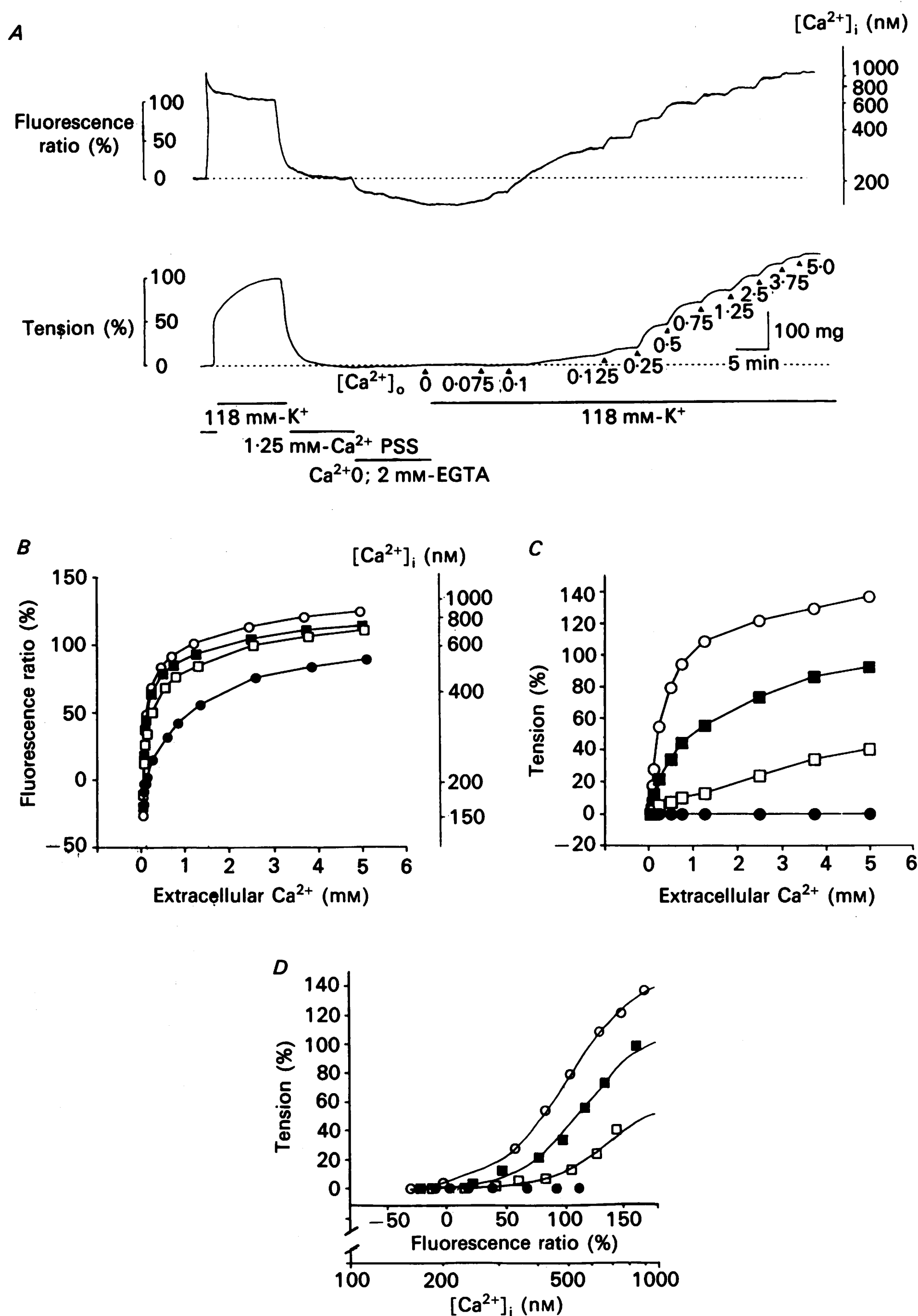


Fig. 5. Effects of caffeine on contraction induced by cumulative addition of extracellular Ca^{2+} during 118 mM-K^+ depolarization. *A*, representative recordings of the fluorescence ratio and the tension obtained in response to the increase in extracellular Ca^{2+} concentration (▲) in the absence of caffeine. *B* and *C*, similar experiments were performed in the presence of various concentrations of caffeine (no caffeine, ○; 2 mM, ■; 5 mM, □;

tension (Fig. 6*A*). Although the fluorescence ratio remained higher than the resting level ($59{\cdot}1 \pm 6{\cdot}7$ %, $[Ca^{2+}]_i = 388$ nM, $n = 4$), the tension returned to or below the resting level. The inhibitory effects of caffeine on NA-induced increases in fluorescence ratio and tension were concentration dependent (Fig. 6*B* and *C*). The

TABLE 1. Parameters for $[Ca^{2+}]_i$–tension curves in Fig. 5*D*

[Caffeine] (mM)	K_d (nM)	T_{max} (%)	α
0	$508{\cdot}6 \pm 36{\cdot}9$	$151{\cdot}9 \pm 16{\cdot}5$	$3{\cdot}73 \pm 0{\cdot}48$
2	$557{\cdot}3 \pm 92{\cdot}1$	$109{\cdot}4 \pm 9{\cdot}0$	$4{\cdot}73 \pm 1{\cdot}33$
5	$720{\cdot}1 \pm 195{\cdot}5$	$65{\cdot}2 \pm 16{\cdot}7$	$4{\cdot}05 \pm 1{\cdot}17$

K_d, apparent dissociation constant; T_{max}, maximal tension estimated from Fig. 5*C*; α, Hill coefficient.

IC_{50} value for the inhibition by caffeine of the NA-induced increase in fluorescence ratio and tension was about 20 mM, and about 0·5 mM, respectively.

Effects of dibutyryl cyclic adenosine monophosphate on smooth muscle contraction

As caffeine increases cAMP concentration in vascular smooth muscle cells (Ahn *et al.* 1988), this nucleotide may play a role in the inhibitory effect of caffeine. We examined the effects of dibutyryl cyclic adenosine monophosphate (dBcAMP) on $[Ca^{2+}]_i$ and tension in rat aortic smooth muscle. Figure 7*A* shows the effects of dBcAMP on the 118 mM-K^+ contraction. Application of 10^{-4} M-dBcAMP during the 118 mM-K^+ contraction caused a gradual decrease in the tension to a level of $6{\cdot}0 \pm 3{\cdot}2$ % ($n = 3$) with a small decrease in the fluorescence ratio. In contrast to caffeine, dBcAMP did not cause an initial increase either in fluorescence ratio or in tension. During the 10^{-6} M-NA-induced contraction, 10^{-4} M-dBcAMP reduced the fluorescence ratio to a level slightly higher than the resting level and reduced the tension to below the resting level (Fig. 7*B*). Thus, the extent of reduction in tension was greater than that expected from the reduction in $[Ca^{2+}]_i$, based on the $[Ca^{2+}]_i$–tension relationship observed with K^+ depolarization (Fig. 1*B*).

Effects of caffeine on $^{45}Ca^{2+}$ *influx*

To determine the mechanisms by which caffeine decreases $[Ca^{2+}]_i$, the effects of caffeine on the $^{45}Ca^{2+}$ influx stimulated by 118 mM-K^+ depolarization or NA were examined. These experiments were carried out as follows. First, we measured the unidirectional $^{45}Ca^{2+}$ influx for 2 min in normal PSS, as a control. The second group of strips was exposed to 118 mM-K^+ PSS or 10^{-5} M-NA in normal PSS for 20 min, then incubated for 2 min in 118 mM-K^+ PSS or 10^{-5} M-NA in normal PSS containing $^{45}Ca^{2+}$. The third group was exposed to 118 mM-K^+ PSS or 10^{-5} M-NA in normal PSS for 10 min, then to 118 mM-K^+ or 10^{-5} M-NA containing various concentrations of

10 mM, ●). Caffeine inhibited increases both in fluorescence ratio (*B*) and in tension (*C*), in a concentration-dependent manner. *D*, $[Ca^{2+}]_i$–tension curves in the absence (○) and presence of 2 (■), 5 (□) and 10 mM-caffeine (●). Parameters for each curve are presented in Table 1. Caffeine changed the $[Ca^{2+}]_i$–tension relation so that it was less effective in producing tension at a given $[Ca^{2+}]_i$, in a concentration-dependent manner.

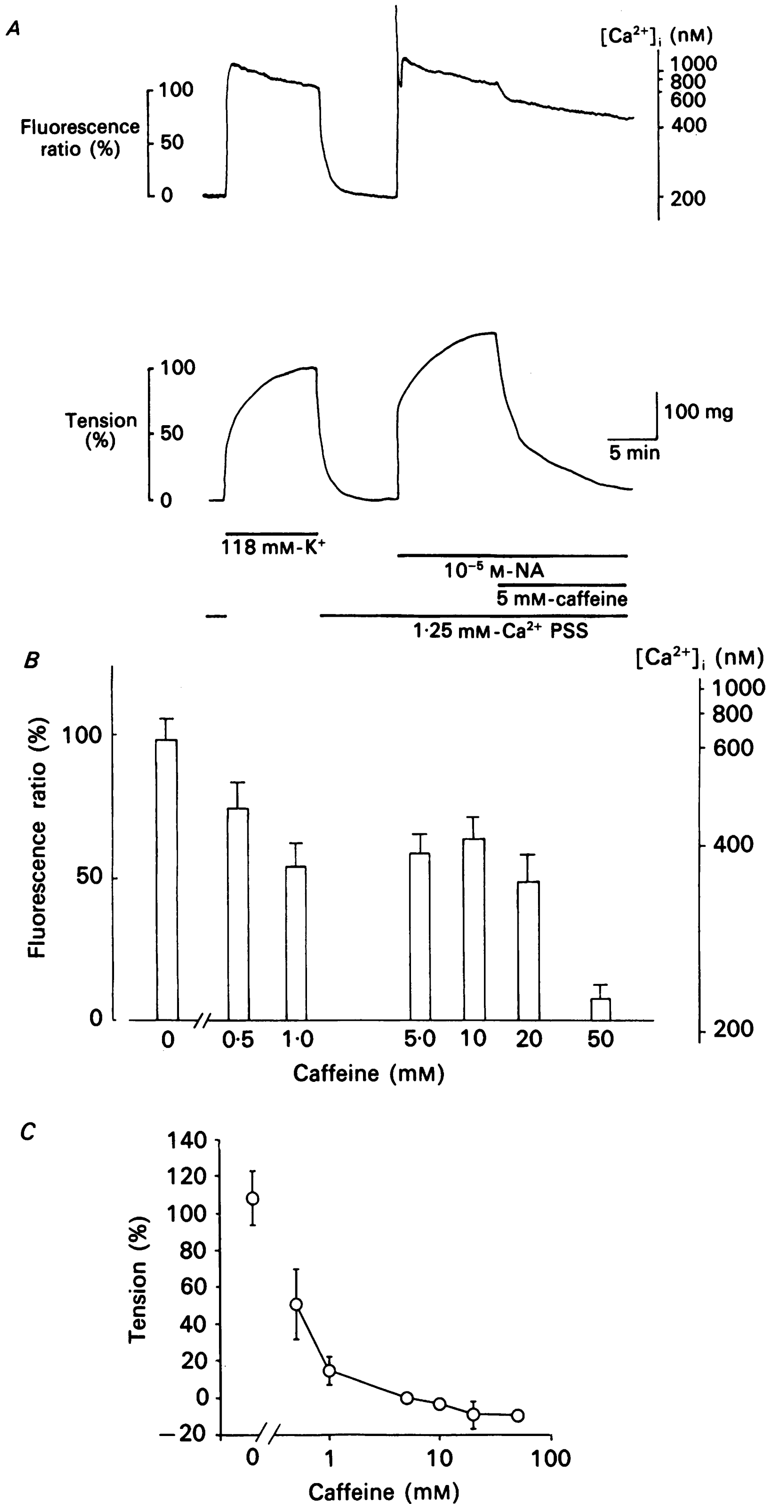


Fig. 6. For legend see facing page.

caffeine for 10 min, and lastly to 118 mM-K^+ PSS or 10^{-5} M-NA in normal PSS, containing caffeine and $^{45}Ca^{2+}$, for 2 min. Finally, the $^{45}Ca^{2+}$ influx was measured in the presence of 10 or 50 mM-caffeine at 10 min after application in normal PSS and without exposure to 118 mM-K^+ PSS or to 10^{-5} M-NA.

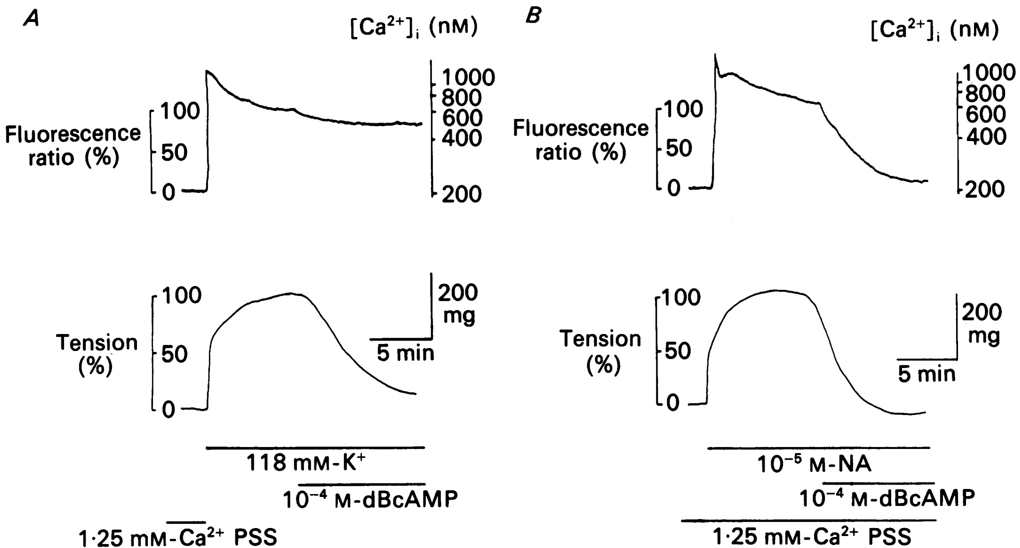


Fig. 7. Effects of dBcAMP on 118 mM-K^+- and NA-induced contraction. *A*, during the 118 mM-K^+-induced contraction ($275{\cdot}3 \pm 12{\cdot}4$ mg), application of 10^{-4} M-dBcAMP inhibited the developed tension to a level of $6{\cdot}0 \pm 3{\cdot}2$% ($n = 3$) (lower trace) with a slight decrease in fluorescence ratio (upper trace). *B*, during NA (10^{-5} M)-induced contraction ($287{\cdot}4 \pm 10{\cdot}2$ mg), application of 10^{-4} M-dBcAMP completely inhibited the developed tension (lower trace). In contrast to 118 mM-K^+ contraction, dBcAMP almost completely inhibited the increase in fluorescence ratio induced by NA.

Calcium influx in normal PSS was $83{\cdot}0 \pm 12{\cdot}7$ μmol (kg wet weight)$^{-1}$ (2 min)$^{-1}$ ($n = 5$) and it increased to $131{\cdot}9 \pm 15{\cdot}5$ μmol kg^{-1} (2 min)$^{-1}$ ($n = 5$) with 118 mM-K^+ depolarization. Caffeine decreased the Ca^{2+} influx induced by 118 mM-K^+ depolarization, in a concentration-dependent manner (Fig. 8*A*, $n = 5$). Caffeine (10 and 50 mM) alone did not significantly alter the Ca^{2+} influx in normal PSS. Thus,

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Fig. 6. Effects of caffeine on noradrenaline (NA)-induced contraction. *A*, representative recordings of the changes in fluorescence ratio and tension elicited by application of 5 mM-caffeine during NA (10^{-5} M)-induced contraction. Tension induced by 118 mM-K^+ was $266 \pm 9{\cdot}2$ mg. *B*, a concentration–response relationship of the inhibitory effect of caffeine on the fluorescence ratio during the NA-induced contraction. The fluorescence ratio was determined at 10 min after the application of various concentrations of caffeine. *C*, a concentration–response curve of the inhibitory effect of caffeine on tension during the NA-induced contraction. The tension was determined at 10 min after the application of various concentrations of caffeine. Caffeine inhibited the increase in fluorescence ratio (IC_{50} of about 20 mM) and the tension development (IC_{50} of about 0·5 mM) induced by NA in a concentration-dependent manner. Data are means ± S.D. from four different experiments.

caffeine inhibited the increase in Ca^{2+} influx with 118 mM-K^+ depolarization. The IC_{50} for the inhibition of Ca^{2+} influx was 21·4 ± 4·3 mM ($n = 5$) and was higher than that for the inhibitory effect on $[Ca^{2+}]_i$ (about 10 mM).

In the same manner, the effects of caffeine on the Ca^{2+} influx during NA activation were examined (Fig. 8*B*). The Ca^{2+} influx increased from 89·8 ± 11·9 to 167·8 ± 32·4 μmol kg^{-1} (2 min)$^{-1}$ ($n = 5$) with 10^{-5} M-NA. Caffeine decreased the Ca^{2+} influx in a concentration-dependent manner with a graphically determined IC_{50} of about 12·5 mM.

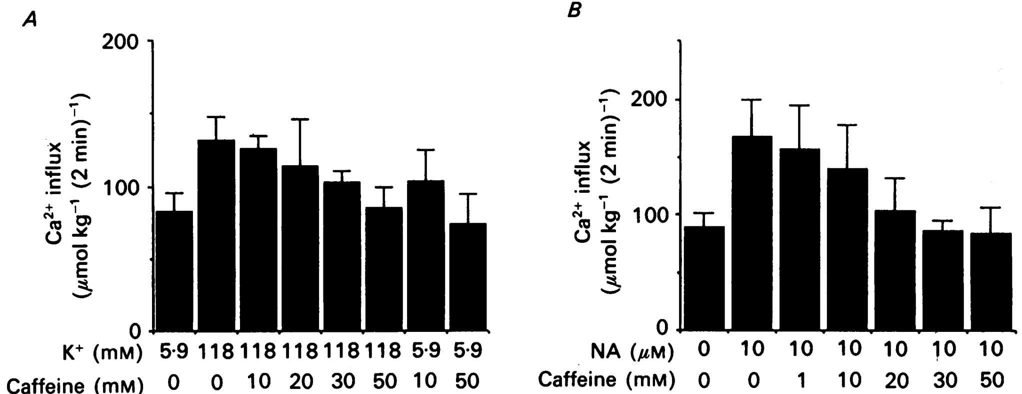

Fig. 8. Effects of caffeine on $^{45}Ca^{2+}$ influx into the strips stimulated with 118 mM-K^+ depolarization (*A*) and 10^{-5} M-NA (*B*). Both 118 mM-K^+ depolarization and NA (10^{-5} M) increased $^{45}Ca^{2+}$ influx by 1·6 and 1·9 times, respectively. Caffeine inhibited the increase in $^{45}Ca^{2+}$ influx induced by high-K^+ depolarization (*A*, IC_{50} = 21·4 ± 4·3 mM) or NA (*B*, IC_{50} = 12·5 mM) in a concentration-dependent manner. Caffeine (10 and 50 mM) alone did not increase $^{45}Ca^{2+}$ influx significantly in normal PSS. Data are means ± S.D. from five different experiments.

DISCUSSION

Our results clearly showed that the relationship between $[Ca^{2+}]_i$ and tension observed during the contraction or relaxation induced by caffeine differed from that of K^+ depolarization; the latter was similar to that seen with skinned tissues. (1) For a given increase in $[Ca^{2+}]_i$, the tension development induced by caffeine (5–50 mM) in normal PSS was much smaller than that induced by 118 mM-K^+ depolarization (Fig. 2*E*). (2) Tension development induced by caffeine (5–50 mM) in normal PSS was transient. Although tension returned to the resting level, $[Ca^{2+}]_i$ was sustained at the level that would induce about 60% of tension development in a K^+-depolarized strip, as estimated from the $[Ca^{2+}]_i$–tension curve in Fig. 2*E*. (3) Caffeine relaxed the vascular strips pre-contracted by high-K^+ depolarization (Figs 3 and 4) or by NA (Fig. 6). Relaxations of the strips were accompanied by decreases in $[Ca^{2+}]_i$, but were more pronounced than those predicted from the extent of decreases in $[Ca^{2+}]_i$. Caffeine at 5 mM completely inhibited the 118 mM-K^+-induced contraction, while it reduced $[Ca^{2+}]_i$ by only 51·5% (Fig. 3). Such a level of $[Ca^{2+}]_i$ would induce about 30% of the contraction in a K^+-depolarized strip, according to the results in Figs 1*B*

and 5*D*. These findings suggest that caffeine may relax smooth muscle not only by decreasing $[Ca^{2+}]_i$ but also by interfering with the relation between $[Ca^{2+}]_i$ and the extent of tension development, namely a process (or processes) in the signal transduction from Ca^{2+} to contractile proteins. Sato *et al.* (1988) reported that the inhibitory effects of caffeine were not only the result of a decrease in $[Ca^{2+}]_i$ but also were related to a direct effect on contractile elements. In the present study, we attempted to clarify the mechanism of this inhibitory action of caffeine on the $[Ca^{2+}]_i$–tension relation, using the protocol of 'Ca^{2+}-induced contraction during K^+ depolarization' (Fig. 5). Caffeine (2–10 mM) inhibited the Ca^{2+}-induced contraction in the depolarized strips in a concentration-dependent manner. In the extreme, in the presence of 10 mM-caffeine, no contraction was observed, despite an increase in fluorescence ratio up to 90% ($[Ca^{2+}]_i$ = 550 nM). A greater decrease in tension in relation to a slight decrease in $[Ca^{2+}]_i$ resulted in a shift of the $[Ca^{2+}]_i$–tension relation curve to the right and downward (Fig. 5*D*). As shown in Table 1, caffeine increased the K_d value, a parameter of the $[Ca^{2+}]_i$–tension curve equivalent to a $[Ca^{2+}]_i$ that would produce half the maximal contraction; this suggests that caffeine reduces the Ca^{2+} sensitivity of the contractile apparatus, possibly by an effect on Ca^{2+}-binding proteins. In addition, as caffeine decreased the T_{max}, this xanthine may inactivate critical factors in the process of signal transduction from cytosolic Ca^{2+} to contractile proteins. Our results are consistent with the findings in skinned smooth muscles from the myometrium of pregnant rats (Savineau & Mironneau, 1990) and from the chicken gizzard (Ozaki *et al.* 1990), where caffeine inhibited Ca^{2+}-induced contractions and shifted Ca^{2+}–tension curves to the right and downward. On the contrary, it was reported that caffeine did not alter pCa–tension curves in skinned smooth muscles from the porcine coronary artery (Itoh, Kajiwara, Kitamura & Kuriyama, 1982) and the guinea-pig mesenteric artery (Itoh *et al.* 1981). Thus, the effect of caffeine may differ depending on the species, tissues and/or method of preparation. Figure 5*D* clearly indicates that with regard to the inhibitory effects of contraction induced by caffeine, the inhibition independent of $[Ca^{2+}]_i$ contributes more than do decreases in $[Ca^{2+}]_i$. It was reported that caffeine increased cAMP concentration in vascular smooth muscle, in a concentration-dependent manner (Ahn *et al.* 1988; Bray, Longmore & Weston, 1989). Cyclic adenosine monophosphate activates protein kinases, which phosphorylate myosin light chain kinase, and more free Ca^{2+} is needed for activation (Adelstein *et al.* 1978). As a result, cAMP may decrease the Ca^{2+} sensitivity of the contractile apparatus (Rüegg *et al.* 1981; Nishimura & van Breemen, 1989). In the present study, 10^{-4} M-dBcAMP actively relaxed the rat aortic smooth muscle pre-contracted by high K^+ or NA (Fig. 7), and the extent of reduction in tension in both cases was greater than that expected from the reduction in $[Ca^{2+}]_i$ based on the $[Ca^{2+}]_i$–tension relationship observed with K^+ depolarization (Fig. 1*B*). Thus, the inhibitory effects of caffeine on the $[Ca^{2+}]_i$–tension relation may be mediated by cAMP. More quantitative studies are needed to confirm the role of cAMP.

To investigate the mechanisms of the caffeine-induced decrease in $[Ca^{2+}]_i$, we examined the effects of caffeine on the influx of $^{45}Ca^{2+}$. Our results showed that caffeine inhibited the increase in $^{45}Ca^{2+}$ influx induced by high-K^+ depolarization, in a concentration-dependent manner. Thus, caffeine blocks voltage-dependent Ca^{2+}

channels. This observation is consistent with the data of Martin, Dacquet, Mironneau & Mironneau (1989) that caffeine inhibits Ca^{2+} currents through voltage-dependent Ca^{2+} channels in rat cultured myometrium, with an IC_{50} of 35 mM. It was reported that NA induced Ca^{2+} influx through voltage-dependent Ca^{2+} channels, via a receptor-mediated second messenger system (Nelson, Standem, Brayden & Worley, 1988). On the other hand, activation of other Ca^{2+} channels (receptor-operated Ca^{2+} channels) by agonists has been proposed (Bolton, 1979; Benham & Tsien, 1987). We found that caffeine inhibited the 10^{-5} M-NA-stimulated $^{45}Ca^{2+}$ influx and the 118 mM-K^+-induced $^{45}Ca^{2+}$ influx to a similar extent and with a similar IC_{50} value. The caffeine-induced relaxation in both NA stimulation and 118 mM-K^+-induced contraction was to a similar extent. However, as shown in Fig. 7, dBcAMP slightly reduced $[Ca^{2+}]_i$ during the 118 mM-K^+-induced contraction, and markedly reduced $[Ca^{2+}]_i$ during the NA-induced contraction. On the other hand, caffeine markedly and significantly reduced $[Ca^{2+}]_i$ during the 118 mM-K^+-induced contraction. Thus, this caffeine-induced reduction in $[Ca^{2+}]_i$ may not be mediated by cAMP. Although we found that caffeine relaxed the K^+-induced and NA-induced contraction with a reduction in $[Ca^{2+}]_i$, this may not be due to a reduction in $[Ca^{2+}]_i$ but rather a cAMP-mediated (and $[Ca^{2+}]_i$ independent) inhibition of tension development which may possibly play a major role in the caffeine-related relaxation. The lack of an initial increase in both the fluorescence ratio and tension in the case of dBcAMP, as was observed in the case of caffeine, indicates that cAMP is involved in the relaxation but not in the contraction induced by caffeine.

Pretreatment with ryanodine, a compound which locks Ca^{2+}-release channels of the SR at the open state (Fleischer, Ogunbunmi, Dixon & Fleer, 1985) and is considered to abolish functions of caffeine-sensitive store sites (Iino, Kobayashi & Endo, 1988) did not alter the inhibitory effect of caffeine on the increase in $[Ca^{2+}]_i$ induced by high-K^+ depolarization (Fig. 4). Therefore, although we cannot rule out participation of the ryanodine-insensitive store sites (Iino *et al.* 1988), we do consider that Ca^{2+} uptake into the SR does not participate in the inhibitory effects of caffeine on $[Ca^{2+}]_i$. The IC_{50} for the inhibition of $^{45}Ca^{2+}$-influx by caffeine in the 118 mM-K^+-depolarized strips was $21{\cdot}4 \pm 4{\cdot}3$ mM and was about twice as high as that for the decrease in $[Ca^{2+}]_i$ (about 10 mM). Therefore, mechanisms other than the inhibition of Ca^{2+} influx, for example stimulation of Ca^{2+} extrusion, may be involved in functions or events by which caffeine decreases $[Ca^{2+}]_i$.

In the presence of extracellular Ca^{2+}, caffeine *per se* caused an initial transient followed by a sustained increase in $[Ca^{2+}]_i$, and a transient tension development. The initial transient increase in $[Ca^{2+}]_i$ and the tension may be due to a release of Ca^{2+} from store sites, possibly through the Ca^{2+} (caffeine)-induced Ca^{2+} release pathway (Endo, 1977, 1985), because this response is resistant to the removal of extracellular Ca^{2+} (Fig. 2*B*) and the EC_{50} for the initial transient increase in $[Ca^{2+}]_i$ (4·9 mM) is close to that for the transient tension development (5·5 mM) (Fig. 2*C* and *D*). The latter sustained increase in $[Ca^{2+}]_i$ induced by caffeine *per se* could be due to influx of extracellular Ca^{2+} stimulated by membrane depolarization (Itoh *et al.* 1982). However, in this study, caffeine at 10 mM, a concentration which produced the maximal sustained increase in $[Ca^{2+}]_i$, did not significantly increase the $^{45}Ca^{2+}$ influx (Fig. 8*A*). Furthermore, diltiazem did not inhibit this component of the increase in

$[Ca^{2+}]_i$. Therefore, it is unlikely that the sustained increase in $[Ca^{2+}]_i$ by caffeine, *per se*, in normal PSS is due to an increase in Ca^{2+} influx through the surface membrane. In addition, as already discussed, when vascular strips were pretreated with NA or K^+ depolarization, caffeine inhibited the Ca^{2+} influx through the membrane. The exact mechanism involved in this sustained increase in $[Ca^{2+}]_i$ by caffeine remains to be determined.

In conclusion, despite continuous application, caffeine induced a transient development of tension with a phasic and sustained elevation of $[Ca^{2+}]_i$ in strips of the rat aorta. Both tension development and the phasic component of $[Ca^{2+}]_i$ increase may be due to release of Ca^{2+} from the intracellular store. The sustained increase in $[Ca^{2+}]_i$ may not be related to the influx of Ca^{2+} through the plasma membrane and is accompanied by suppression of the $[Ca^{2+}]_i$–tension relationship. When vascular strips were pre-contracted with NA or high-K^+ depolarization, caffeine induced a relaxation with a reduction in Ca^{2+} influx through the plasma membrane; however, the extent of $[Ca^{2+}]_i$ reduction was not directly related to the extent of relaxation, and there was a marked suppression in the $[Ca^{2+}]_i$–tension relationship. Thus, independent of $[Ca^{2+}]_i$ change, cAMP may directly influence the $[Ca^{2+}]_i$–tension relationships and play a major role in the transiency of contraction and relaxation induced by caffeine in vascular strips.

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