Journal of Physiology (1992), **457**, *pp.* 297–314
With 8 figures
Printed in Great Britain

CHARACTERISTIC FEATURES OF NORADRENALINE-INDUCED Ca^{2+} MOBILIZATION AND TENSION IN ARTERIAL SMOOTH MUSCLE OF THE RABBIT

BY TAKEO ITOH, JUNKO KAJIKURI AND HIROSI KURIYAMA

From the Department of Pharmacology, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

(*Received 4 December 1991*)

SUMMARY

1. Effects of noradrenaline (NAd) on changes in cellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension were investigated, and these effects were compared with those evoked by 128 mM K^+ or caffeine in intact smooth muscle strips or by inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) or caffeine in β-escin-treated chemically skinned smooth muscle strips of the rabbit mesenteric artery.

2. In physiological solution containing 2·6 mM Ca^{2+}, application of 128 mM K^+ or 10 μM NAd produced a phasic, followed by a tonic increase in $[Ca^{2+}]_i$ and tension. NAd (10 μM) produced a larger tonic tension than did 128 mM K^+ but a smaller increase in $[Ca^{2+}]_i$. When the $[Ca^{2+}]_i$–tension relationship was observed in ionomycin- and 128 mM K^+-treated muscle strips, 10 μM NAd shifted the relationship to the left and enhanced the maximum amplitude of contraction. These results suggest that NAd increases the sensitivity of contractile proteins to Ca^{2+} in smooth muscle of the rabbit mesenteric artery.

3. Noradrenaline (10 μM) or caffeine (10 mM), but not 128 mM K^+, produced a phasic increase in both $[Ca^{2+}]_i$ and tension in Ca^{2+}-free solution containing 2 mM EGTA. When 10 mM caffeine had been applied in Ca^{2+}-free solution, subsequent application of 10 μM NAd did not increase $[Ca^{2+}]_i$. By contrast, when 10 μM NAd had been applied in Ca^{2+}-free solution, subsequent application of 10 mM caffeine still increased $[Ca^{2+}]_i$. Ryanodine (50 μM) abolished the increase in $[Ca^{2+}]_i$ induced by 10 mM caffeine or 10 μM NAd in intact and in skinned smooth muscle strips. These results suggest that NAd releases Ca^{2+} from the ryanodine-sensitive Ca^{2+} storage sites.

4. Noradrenaline (10 μM) synthesized Ins(1,4,5)P_3 in Ca^{2+}-free solution in intact smooth muscle strips. Following application of 10 μM NAd, a relatively long time lag (around 1 s) was always observed before the initiation of the increase in $[Ca^{2+}]_i$ whether in the presence or absence of Ca^{2+}. The maximum rate of rise of $[Ca^{2+}]_i$ induced by 10 mM caffeine was much larger than that induced by 10 μM NAd in Ca^{2+}-containing or Ca^{2+}-free solution (containing 2 mM EGTA). Both $[Ca^{2+}]_i$ and tension reached their peak in a shorter time with caffeine (10 mM) than with 10 μM NAd. In β-escin-treated skinned smooth muscle strips, 20 μM Ins(1,4,5)P_3, 10 mM caffeine or 10 μM NAd increased Ca^{2+} in Ca^{2+}-free solution following brief application of 0·3 μM

Ca^{2+}. The extent of the increase in Ca^{2+} was in the rank order Ins(1,4,5)P_3 > caffeine = NAd in identical conditions in terms of Ca^{2+} loading in the storage sites. The time required for $[Ca^{2+}]_i$ to reach its peak was shorter for 20 μM Ins(1,4,5)P_3 or 10 mM caffeine than for 10 μM NAd. These results suggest that NAd may require a certain time lag for α-receptor–phospholipase C coupling to synthesize Ins(1,4,5)P_3 before it can release Ca^{2+}.

5. The rate of decline of $[Ca^{2+}]_i$ in the presence of 10 μM NAd or 10 mM caffeine depended on the concentration of $[Ca^{2+}]_i$ whether in the presence or absence of extracellular Ca^{2+}. The maximum rates of decline of $[Ca^{2+}]_i$ were almost the same for these two agents, but the rate of decline of $[Ca^{2+}]_i$ at lower $[Ca^{2+}]_i$ (150–350 nM) was faster in the presence of NAd than in the presence of caffeine. NAd also enhanced the rate of decline of $[Ca^{2+}]_i$ in the response to 1 μM ionomycin in Ca^{2+}-free solution but to a lesser extent. The maximum rate of decline of $[Ca^{2+}]_i$ in the presence of NAd with ionomycin was almost one-third that observed with NAd alone. These results suggest that, in smooth muscle cells, NAd promotes the process which restores $[Ca^{2+}]_i$ to the resting level through activation of both the re-sequestration of Ca^{2+} and the extrusion of Ca^{2+} to the extracellular space.

6. It is concluded that, in smooth muscle of the rabbit mesenteric artery, NAd releases Ca^{2+} from the ryanodine-sensitive storage site, possibly through the action of Ins(1,4,5)P_3. This agonist negatively controls the increased $[Ca^{2+}]_i$ through activation of Ca^{2+} removal mechanisms. NAd also enhances the sensitivity of contractile proteins to Ca^{2+} and thus increases the tension at lower $[Ca^{2+}]_i$ levels.

INTRODUCTION

The contraction–relaxation cycle in vascular smooth muscle is largely dependent on the cellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) and the sources of Ca^{2+} contributing to the activation of the contractile proteins are thought to be of both extracellular and intracellular origin (Kuriyama, Ito, Suzuki, Kitamura & Itoh, 1982). In smooth muscle of the rabbit mesenteric artery, noradrenaline (NAd) in a low concentration (0·1 μM) produces contraction with no change in the membrane potential ('pharmaco-mechanical coupling'), whereas at high concentrations (over 1 μM), it both depolarized the membrane and caused contraction (Itoh, Kuriyama & Suzuki, 1983). The NAd-induced contraction has a phasic and a subsequent tonic component and the finding that the phasic contraction can be evoked in Ca^{2+}-free solution containing 2 mM EGTA suggests that it may be due to NAd-induced release of Ca^{2+} from its intracellular storage sites (Itoh *et al.* 1983).

In the rabbit mesenteric artery, Ca^{2+} release by NAd is thought to be mediated by inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3), since NAd synthesizes Ins(1,4,5)P_3 through the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) in intact smooth muscle and Ins(1,4,5)P_3 produces contraction in the saponin-treated skinned smooth muscle (Hashimoto, Hirata, Itoh, Kanmura & Kuriyama, 1986). By contrast, caffeine is supposed to act directly on the Ca^{2+} storage sites and release Ca^{2+} due to activation of the Ca^{2+}-induced Ca^{2+} release mechanism (Itoh *et al.* 1983; Leitzen & van Breemen, 1984; Itoh, Kanmura & Kuriyama, 1985). In saponin-skinned smooth muscle strips of the guinea-pig taenia caeci, it has recently been suggested that the Ins(1,4,5)P_3-sensitive Ca^{2+} storage sites are not entirely the same

population as the caffeine-sensitive storage sites since, whereas Ins(1,4,5)P_3 releases almost all the releasable Ca^{2+} from the storage sites, caffeine releases only part of the stored Ca^{2+} (Iino, 1990). At present, it is uncertain whether or not the NAd-sensitive Ca^{2+} storage site is the same as the caffeine-sensitive one.

In human platelets, Rink & Sage (1987) found that thrombin accelerates the decline of $[Ca^{2+}]_i$ after application of ionomycin, suggesting that this agonist stimulates the extrusion of $[Ca^{2+}]_i$ from the cells. Since the complete substitution of extracellular Na^+ with *N*-methyl-D-glucamine did not alter the time course of the return of $[Ca^{2+}]_i$ to the basal level following stimulation by thrombin, they suggested that thrombin accelerates the decline of $[Ca^{2+}]_i$ by stimulation of the Ca^{2+} pump on the plasma membrane. The Ca^{2+} pump on the plasma membrane is also found in smooth muscle (Eggermont, Vrolix, Raeymaekers, Wuytack & Casteels, 1988); however, the action of NAd on the rate of decline of $[Ca^{2+}]_i$ following stimulation has not been clarified in the rabbit mesenteric artery.

It has been reported in the ferret portal vein that, while phenylephrine only transiently increases $[Ca^{2+}]_i$, it produces a maintained contraction (Morgan & Morgan, 1984). Subsequently, it was found that, in skinned vascular smooth muscle strips treated with α-toxin, phenylephrine enhanced the Ca^{2+} sensitivity of the contractile proteins (Nishimura, Kolber & van Breemen, 1988; Kitazawa, Kobayashi, Horiuti, Somlyo & Somlyo, 1989). Moreover, we recently reported that NAd with GTP enhanced the amplitude of contraction induced by 0·3 μM Ca^{2+} in β-escin-treated skinned smooth muscle strips of the rabbit mesenteric artery (Itoh, Suzuki & Kuriyama, 1991). These results suggest that, in intact vascular smooth muscle of the rabbit mesenteric artery, NAd may enhance the sensitivity of contractile proteins to Ca^{2+} and thus produce a given level of contraction at lower concentrations of $[Ca^{2+}]_i$. However, this action of NAd has not been well studied in intact smooth muscle of the rabbit mesenteric artery.

To clarify the characteristic features of Ca^{2+} mobilization and tension induced by NAd in smooth muscle of the rabbit mesenteric artery, we studied the effects of NAd on changes in $[Ca^{2+}]_i$ and tension, and on the relationship between $[Ca^{2+}]_i$ and tension. Further, the actions of NAd on Ca^{2+} release were pharmacologically characterized in detail in intact and β-escin-treated skinned smooth muscle strips.

METHODS

Male albino rabbits, weighing 1·9–2·5 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg/kg, I.V.) and then exsanguinated. The third branch of the mesenteric artery was excised immediately and cleaned by removal of connective tissue in Krebs solution at room temperature.

To enable simultaneous recording of isometric tension and $[Ca^{2+}]_i$, fine circularly cut strips (0·3–0·5 mm length, 0·04–0·05 mm width, 0·02–0·03 mm thickness) were prepared as previously described (Itoh *et al.* 1983). Endothelial cells were removed by gentle rubbing of the internal surface of the vessels using small knives. The absence of the functions of endothelial cells was confirmed by the inability of acetylcholine (1 μM) or the calcium ionophore A23187 (0·1 μM) to cause relaxation during contraction induced by NAd. The strip was transferred into a chamber of 0·1 ml volume and mounted horizontally on an inverted microscope (Diaphot TMD with special optics for epifluorescence, Nikon). The length, width and thickness and the cross-sectional area of the preparation were measured with the inverted microscope as described previously (Itoh, Kanmura & Kuriyama, 1986). All experiments were carried out at room temperature (20–23 °C).

To load Fura-2 into the smooth muscle cells of the strip, 1 μM of the acetoxy methyl ester of

Fura-2 (Fura-2 AM) dissolved by dry dimethyl sulphoxide (1 mM stock solution) was applied for 1 h in Krebs solution at room temperature (20–23 °C). After this period, the solution containing Fura-2 AM was washed out by Krebs solution for over 2 h under dark conditions to ensure sufficient de-esterification of Fura-2 AM in the cells. The position of the strip was adjusted to the centre of the field and a mask placed in an intermediate image plane to reduce the background fluorescence (0·04 mm square). The Fura-2 fluorescence emission at 510 nm using an interference filter (centred at 510 nm with full width at half-transmission of 20 nm) was passed through the objective lens (20 × fluor, Nikon) and collected in a photomultiplier tube (R928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM 400, Nikon) which was substituted for the photochanger in a Nikon Diaphot TMD microscope. Two alternative excitation wavelengths, 340 and 380 nm (each slit 5 nm) were applied by a spectrofluorimeter (Spex, NJ, USA) and the data analysed using customized software provided by Spex (DM-3000CM).

The ratio of the Fura-2 fluorescence intensities excited by 340 and 380 nm UV light was calculated after subtraction of the background fluorescence. Background fluorescence (including the autofluorescence of the strip) as excited by 340 and 380 nm UV light was measured following application of a solution containing 50 μM ionomycin, 20 mM $MnCl_2$, 110 mM KCl and 10 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS) (pH 4·8) after the experiment, as previously described by Himpens, Matthijs & Somlyo (1989). Under these conditions, the background fluorescence intensity was 10–15% of the Fura-2 signals in smooth muscle strips at either excitation wavelength. Cytosolic Ca^{2+} concentrations were calculated using the formula described by Grynkiewicz, Poenie & Tsien (1985) and *in vitro* calibration (Poenie, Alderton, Steinhart & Tsien, 1986; Becker, Singer, Walsh & Fay, 1989). The ratio of maximum (F_{max}) to minimum fluorescence (F_{min}) was determined in the calibration solution after subtraction of background and the 380 nm signal of Fura-2 was assumed to decrease by 15% in the cell due to the possible intracellular viscosity effects of the dye (Poenie *et al.* 1986; Becker *et al.* 1989). The K_d value for Fura-2 was estimated to be 200 nM (Becker *et al.* 1989).

To enable observation of the relationship between $[Ca^{2+}]_i$ and tension in intact smooth muscle strips, the membrane was depolarized by application of a Ca^{2+}-free solution containing 128 mM K^+ with 2 mM EGTA, and Ca^{2+} (0·08–2·6 mM) was cumulatively applied from low to high concentration. To prevent overflow of NAd from sympathetic nerve terminals and release of Ca^{2+} from storage sites during application of Ca^{2+}, 3 μM guanethidine and 1 μM ionomycin were applied throughout this experiment.

Experiments on chemically skinned smooth muscle

Chemically skinned smooth muscle strips were made using β-escin (Kobayashi, Kitazawa, Somlyo & Somlyo, 1989). The methods used to make skinned muscles and the composition of the solution have been described elsewhere (Itoh *et al.* 1986; Kobayashi *et al.* 1989; Itoh *et al.* 1991). To enable measurement of the Ca^{2+} released from its storage sites, 0·3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min (to load Ca^{2+} into the storage sites) and Ca^{2+} was removed from the solution by application of Ca^{2+}-free solution containing 4 mM EGTA for 0·5 min. Then, a solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2 was applied for 2 min. Finally, 10 μM NAd, 10 mM caffeine or 10 μM Ins(1,4,5)P_3 was applied for 1 min in a solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2.

Measurement of Ins(1,4,5)P_3

Endothelium-denuded strips (10 mm length, 2·2–2·5 mm width, 0·1 mm thickness) were equilibrated for over 2 h at 25 °C in Krebs solution. After this, the strips were transferred to Ca^{2+}-free Krebs solution containing 2 mM EGTA for 2 min and then 10 μM NAd was applied for 5 or 60 s. The reaction was stopped by addition of a large amount of ice-cold trichloroacetic acid (final concentration 8%) and the strips then homogenized. The homogenate was centrifuged and the supernatant fraction treated with ether three times and assayed using a radioimmunoassay kit from Amersham International. To minimize the loss of Ins(1,4,5)P_3, a Teflon tube was used instead of glassware after homogenization.

Solutions

The ionic composition of the Krebs solution was as follows (mM): Na^+, 137·4; K^+, 5·9; Mg^{2+}, 1·2; Ca^{2+}, 2·6; HCO_3^-, 15·5; $H_2PO_4^-$, 1·2; Cl^-, 134; glucose, 11·5. The concentration of K^+ was modified by replacing NaCl with KCl, isosmotically. To prevent NAd outflow from sympathetic nerve

terminals, 3 μM guanethidine was added to the Krebs solution throughout the experiment. Ca^{2+}-free solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ and adding 2 mM EGTA. The solutions were bubbled with 95% O_2 and 5% CO_2, and their pH maintained at 7·3–7·4.

The calibration solution for Ca^{2+} measurement contained 11 mM EGTA, 110 mM KCl, 1 mM $MgCl_2$, 2 μM Fura-2 and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (pH 7·1) with or without 11 mM $CaCl_2$.

For experiments on skinned muscle, the composition of the relaxing solution was: 87 mM potassium methanesulphonate (KMS), 20 mM piperazine-*N*-*N'*-bis-(2-ethanesulphonic acid) (PIPES), 5·1 mM $Mg(MS)_2$, 5·2 mM ATP, 10 mM phosphocreatine and 4 mM ethyleneglycol-bis-(*β*-aminoethylether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA). To enable measurement of Ca^{2+} release from skinned strips, the concentration of EGTA was reduced to 50 μM and 2 μM Fura-2 added (as described above). Various Ca^{2+} concentrations were prepared by adding appropriate amounts of $Ca(MS)_2$ to 4 mM EGTA, based on the calculation reported previously (Itoh *et al.* 1986). The pH of the solution was adjusted to 7·1 at 25 °C with KOH and the ionic strength standardized at 0·2 M by changing the amount of KMS added.

Drugs

Drugs used were Fura-2, Fura-2 AM, EGTA, PIPES, HEPES and MOPS (Dojin, Japan), NAd, Ins(1,4,5)P_3, GTP and *β*-escin (Sigma), guanethidine (Tokyo Kasei, Japan), ATP (sodium salt; Kojin, Japan) and ionomycin (free acid; Calbiochem).

Statistics

The values recorded were expressed as means ± S.D., and statistical significance determined using Student's *t* test. Probabilities less than 5% ($P < 0·05$) were considered significant.

RESULTS

General features of changes in $[Ca^{2+}]_i$ and tension induced by 10 μM NAd, 10 mM caffeine or 128 mM K^+

Figure 1 shows traces of the effects of 128 mM K^+, 10 μM NAd and 10 mM caffeine on $[Ca^{2+}]_i$ and tension in Ca^{2+}-containing (2·6 mM) or Ca^{2+}-free (containing 2 mM EGTA) solution in a smooth muscle strip of the rabbit mesenteric artery. The resting $[Ca^{2+}]_i$ was 91·6 ± 11·1 nM in Ca^{2+}-containing solution and 73·1 ± 7·9 nM in Ca^{2+}-free solution ($n = 10$). Each stimulus was applied for 2 min at 20 min intervals. In Ca^{2+}-containing solution, 128 mM K^+ produced a large phasic, followed by a tonic increase in $[Ca^{2+}]_i$ and tension. The phasic increases in $[Ca^{2+}]_i$ and tension induced by 128 mM K^+ took the levels respectively to 497·7 ± 153·6 nM and 44·1 ± 9·8 kN/m^2, and the tonic increases to 207·3 ± 41·9 nM and 16·7 ± 8·3 kN/m^2 ($n = 10$). By contrast, following 2 min removal of Ca^{2+} by application of Ca^{2+}-free solution containing 2 mM EGTA, 128 mM K^+ failed to increase either $[Ca^{2+}]_i$ or tension (Figs 1*B* and 2*B*).

In Ca^{2+}-containing solution, 10 μM NAd produced large phasic increases in $[Ca^{2+}]_i$ and tension, followed by a relatively large tonic increase in tension with a small increase in $[Ca^{2+}]_i$. The phasic increases in $[Ca^{2+}]_i$ and tension induced by 10 μM NAd took the levels respectively to 448·4 ± 165·7 nM and 39·2 ± 6·9 kN/m^2, and the tonic increases to 138·7 ± 21·3 nM and 27·0 ± 12·3 kN/m^2 ($n = 10$). In Ca^{2+}-free solution containing 2 mM EGTA, 10 μM NAd transiently increased $[Ca^{2+}]_i$ (to 406·1 ± 68·1 nM) and tension (to 46·1 ± 4·4 kN/m^2, $n = 6$). The increases in $[Ca^{2+}]_i$ and tension induced by 10 μM NAd were abolished by 1 μM prazosin in the presence or absence of extracellular Ca^{2+}.

Caffeine transiently increased $[Ca^{2+}]_i$ and tension in Ca^{2+}-containing or Ca^{2+}-free solution. The maximum increases in $[Ca^{2+}]_i$ and tension induced by 10 mM caffeine took the levels respectively to $507{\cdot}2 \pm 151{\cdot}2$ nM and $38{\cdot}2 \pm 9{\cdot}3$ kN/m^2 in Ca^{2+}-containing solution and to $483{\cdot}1 \pm 60{\cdot}8$ nM and $40{\cdot}2 \pm 7{\cdot}8$ kN/m^2 in Ca^{2+}-free solution ($n = 7$).

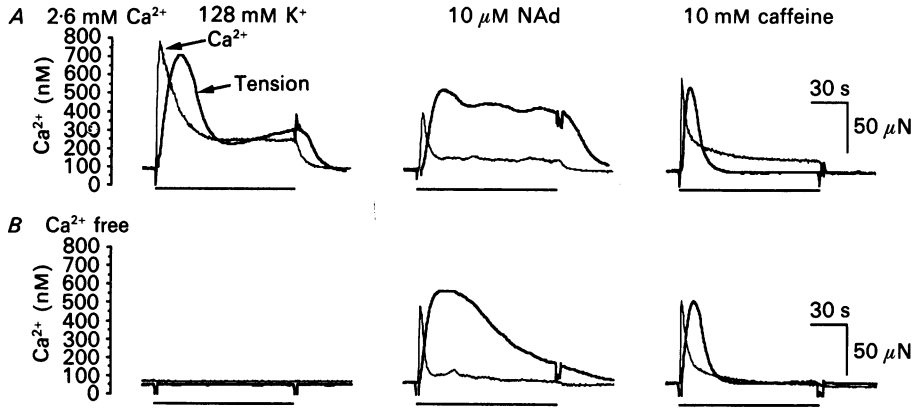

Fig. 1. Effects of 128 mM K^+, 10 μM NAd or 10 mM caffeine on $[Ca^{2+}]_i$ and tension in a smooth muscle strip of the rabbit mesenteric artery in the presence or absence of extracellular Ca^{2+}. *A*, in the presence of 2·6 mM Ca^{2+}. *B*, in Ca^{2+}-free solution containing 2 mM EGTA. Individual stimuli were applied for 2 min at 20 min intervals. Thinner and thicker traces indicate $[Ca^{2+}]_i$ and tension, respectively. The stimulus was applied where indicated by the bar. In *B*, the stimuli were applied 2 min after application of Ca^{2+}-free solution containing 2 mM EGTA.

Figure 2 shows traces of the rates of rise and fall of $[Ca^{2+}]_i$ and tension as a function of time following application of 128 mM K^+, 10 μM NAd or 10 mM caffeine in a smooth muscle strip of the rabbit mesenteric artery. The tension evoked by individual stimuli always developed after the peak rate of rise of $[Ca^{2+}]_i$, suggesting that the increased $[Ca^{2+}]_i$ evoked by the stimulus initiates the tension development. The maximum values for the rate of rise of $[Ca^{2+}]_i$ induced by 128 mM K^+, 10 μM NAd and 10 mM caffeine were $398{\cdot}5 \pm 148{\cdot}9$, $252{\cdot}1 \pm 156{\cdot}8$ and $487{\cdot}0 \pm 132{\cdot}4$ nM/s, respectively ($n = 7$–10). The maximum values for the rate of rise of tension induced by 128 mM K^+, 10 μM NAd and 10 mM caffeine were $25{\cdot}5 \pm 7{\cdot}8$, $14{\cdot}7 \pm 6{\cdot}9$ and $24{\cdot}5 \pm 11{\cdot}8$ μN/s, respectively ($n = 7$–10). Thus, for these three stimulants, the rank order for the maximum rate of rise of $[Ca^{2+}]_i$ coincides with that for the maximum rate of rise of tension. The delay between the occurrence of the maximum rate of rise of $[Ca^{2+}]_i$ and that of tension was almost the same for the three stimulants ($2{\cdot}2 \pm 0{\cdot}7$ s for 128 mM K^+, $3{\cdot}0 \pm 1{\cdot}4$ s for 10 μM NAd and $2{\cdot}3 \pm 0{\cdot}9$ s for 10 mM caffeine, $n = 7$–10).

Initiation of the NAd-induced increase in $[Ca^{2+}]_i$ occurred after a certain time lag whether in the presence or absence of extracellular Ca^{2+} (for example, Fig. 5*A* and *C*). The times required for $[Ca^{2+}]_i$ to reach its maximum rate of rise after application of 128 mM K^+, 10 μM NAd and 10 mM caffeine were $1{\cdot}9 \pm 0{\cdot}5$, $3{\cdot}7 \pm 0{\cdot}7$ and $2{\cdot}1 \pm 0{\cdot}8$ s, respectively ($n = 7$–10). The corresponding times required for tension to reach its maximum rate of rise were $4{\cdot}1 \pm 0{\cdot}9$, $6{\cdot}7 \pm 1{\cdot}4$ and $4{\cdot}4 \pm 1{\cdot}4$ s, respectively ($n = 7$–10).

Thus, the time required for both $[Ca^{2+}]_i$ and tension to reach their peak rate of rise after application of the stimuli was significantly longer for 10 μM NAd than for the other stimulants (Fig. 2*Bb*, $P < 0.05$).

Noradrenaline and caffeine release Ca^{2+} from the ryanodine-sensitive Ca^{2+} storage sites

To help characterize the NAd- and caffeine-sensitive cellular Ca^{2+} storage sites, the effects of pre-application of 10 μM NAd on the increase in $[Ca^{2+}]_i$ induced by 10 mM

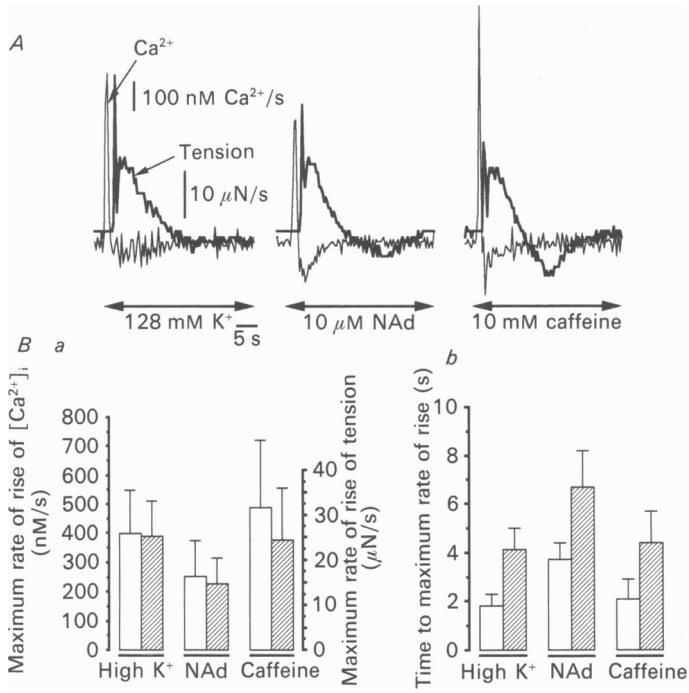

Fig. 2. *A*, changes in the rates of rise and fall of $[Ca^{2+}]_i$ and tension as a function of time after application of 128 mM K^+, 10 μM NAd or 10 mM caffeine in a smooth muscle strip of the rabbit mesenteric artery. Individual stimuli were applied for 2 min at 20 min intervals in Krebs solution (containing 2·6 mM Ca^{2+}). The thinner and thicker traces are $[Ca^{2+}]_i$ and tension, respectively. The stimulants were applied as indicated by ↔. The rates of change in $[Ca^{2+}]_i$ and tension were calculated at 0·4 s intervals. *B*, the maximum rates of rise of $[Ca^{2+}]_i$ and tension induced by high K^+ (128 mM), NAd (10 μM) or caffeine (10 mM) in smooth muscle strips of the rabbit mesenteric artery. In *Ba* □, $[Ca^{2+}]_i$ (nM/s); ▨, tension (μN/s). The rates of change for $[Ca^{2+}]_i$ and tension were calculated at 0·4 s intervals. *Bb*, the time for $[Ca^{2+}]_i$ and tension to reach their maximum rate of rise in the presence of 128 mM K^+, 10 μM NAd or 10 mM caffeine. The individual stimuli were applied 2 min after application of Ca^{2+}-free solution containing 2 mM EGTA. □, $[Ca^{2+}]_i$; ▨, tension. Results shown are each the mean of seven to ten observations with S.D. shown by a vertical bar.

caffeine were studied in Ca^{2+}-free solution containing 2 mM EGTA (Fig. 3*A*). Following 2 min removal of Ca^{2+}, 10 μM NAd was applied twice for 2 min with a 3 min interval to mobilize the Ca^{2+} stored in the smooth muscle strips. When 10 μM NAd was repetitively applied in Ca^{2+}-free solution, the increase in $[Ca^{2+}]_i$ induced by

the second application of NAd was much smaller than that evoked by the first. When 10 mM caffeine was applied 3 min after the second application of 10 μM NAd in Ca^{2+}-free solution, caffeine still increased $[Ca^{2+}]_i$ and this response was larger than that evoked by the second application of NAd (Fig. 3*A*).

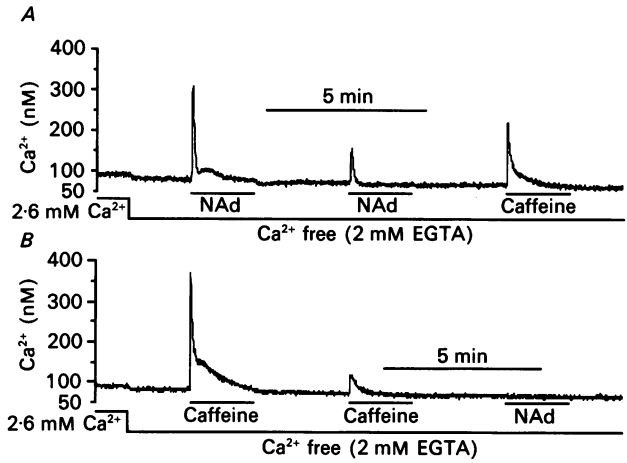

Fig. 3. Effects of pre-application of 10 μM NAd (*A*) or 10 mM caffeine (*B*) on change in $[Ca^{2+}]_i$ induced by 10 μM NAd in Ca^{2+}-free solution containing 2 mM EGTA in a smooth muscle strip of the rabbit mesenteric artery. *A*, after 2 min application of Ca^{2+}-free solution containing 2 mM EGTA, 10 μM NAd was twice applied for 2 min with a 3 min interval and 10 mM caffeine then applied for 2 min following 3 min wash-out of NAd by Ca^{2+}-free solution. *B*, after removal of Ca^{2+} for 2 min, 10 mM caffeine was twice applied for 2 min with a 3 min interval, and 10 μM NAd then applied for 2 min following 3 min wash-out of caffeine.

When 10 mM caffeine was repetitively applied for 2 min with a 3 min interval in Ca^{2+}-free solution, the amplitude of the increase in $[Ca^{2+}]_i$ induced by the second application of caffeine was greatly reduced and a subsequent application of 10 μM NAd failed to increase $[Ca^{2+}]_i$ (Fig. 3*B*).

Ryanodine, a neutral plant alkaloid, binds to the membrane of the sarcoplasmic reticulum (SR) in skeletal muscle and locks the Ca^{2+}-release channel open in cardiac and skeletal muscle (Fleischer, Ogunbunmi, Dixon & Fleer, 1985; Rousseau, Smith & Meissner, 1987). To investigate whether or not NAd and caffeine release Ca^{2+} from the ryanodine-sensitive storage sites, the effects of ryanodine on the increases in $[Ca^{2+}]_i$ and tension induced by NAd and caffeine were observed in smooth muscle strips of the rabbit mesenteric artery.

The effects of 50 μM ryanodine were studied on the increase in $[Ca^{2+}]_i$ induced by 10 mM caffeine or 10 μM NAd in Krebs solution (containing 2·6 mM Ca^{2+}). NAd (10 μM) or caffeine (10 mM) were applied at 10 min intervals in Krebs solution in the absence of ryanodine, and 50 μM ryanodine with 10 mM caffeine was applied for 10 min. After 10 min wash-out of caffeine, 10 μM NAd or 10 mM caffeine was applied in the presence of 50 μM ryanodine in Krebs solution. The increases in $[Ca^{2+}]_i$ and tension induced by 10 μM NAd or 10 mM caffeine were greatly reduced in the presence

of 50 μM ryanodine. The inhibition induced by 50 μM ryanodine on increases in $[Ca^{2+}]_i$ and in tension induced by 10 μM NAd were respectively 12·0 ± 3·2 and 14·5 ± 3·8% of the control ($n = 4$), and the effects on increases in $[Ca^{2+}]_i$ and in tension induced by 10 mM caffeine were respectively 15·2 ± 5·8 and 7·8 ± 5·2% of the control ($n = 4$).

Effects of NAd, Ins(1,4,5)P_3 and caffeine on Ca^{2+} release from the storage sites in β-escin-skinned smooth muscle

In Ca^{2+}-free solution containing 2 mM EGTA, 10 μM NAd transiently increased Ins(1,4,5)P_3 within 5 s and it then decreased to the resting level after 1 min. The concentration of Ins(1,4,5)P_3 was 15·2 ± 6·8 pmol/mg protein in the resting condition, 35·8 ± 8·2 pmol/mg protein at 5 s ($P < 0·05$, compared with the resting value, $n = 5$) and 16·8 ± 8·2 pmol/mg protein at 1 min ($P > 0·05$, $n = 5$) after application of 10 μM NAd.

To help characterize the NAd-induced Ca^{2+} release further, the effects of NAd, caffeine and Ins(1,4,5)P_3 on the release of Ca^{2+} from its storage sites were observed in β-escin-treated skinned smooth muscle strips (Fig. 4). After the strips were skinned by application of 20 μM β-escin for 25 min, 0·3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min to load Ca^{2+} into the storage sites, and Ca^{2+}-free solution containing 4 mM EGTA was applied for 0·5 min to remove Ca^{2+} from the solution. Finally, 10 μM NAd, 10 mM caffeine or 20 μM Ins(1,4,5)P_3 was applied for 1 min in Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2 following application of Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2 for 2 min.

In Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2, the resting Ca^{2+} concentration was 40–80 nM. Under the conditions of our experiment, 10 μM NAd, 10 mM caffeine and 20 μM Ins(1,4,5)P_3 each increased Ca^{2+}, possibly due to release of Ca^{2+} from its storage sites (Fig. 4*A*). When the extent of each increase in Ca^{2+} induced by the three agents was expressed as 'ΔCa^{2+}' (the peak Ca^{2+} − resting Ca^{2+}), the values were 26·4 ± 10·0 nM for 10 μM NAd, 28·1 ± 8·4 nM for 10 mM caffeine and 48·0 ± 10·6 nM for 20 μM Ins(1,4,5)P_3. The evoked maximum rate of rise of Ca^{2+} was 33·7 ± 13·4 nM/s for 10 μM NAd, 37·0 ± 3·2 nM/s for 10 mM caffeine and 55·2 ± 19·2 nM/s for 20 μM Ins(1,4,5)P_3 ($n = 5$–9). The times required for Ca^{2+} to reach its peak after application of 10 μM NAd, 10 mM caffeine and 20 μM Ins(1,4,5)P_3 were 3·9 ± 1·3, 2·5 ± 0·8 and 2·1 ± 0·9 s, respectively ($n = 5$–9). Prazosin (1 μM) inhibited the increase in Ca^{2+} induced by 10 μM NAd, but not that induced by 10 mM caffeine or 20 μM Ins(1,4,5)P_3.

We investigated the effects of ryanodine on the increases in Ca^{2+} induced by 10 mM caffeine, 10 μM NAd and 20 μM Ins(1,4,5)P_3 in β-escin-treated skinned smooth muscle strips in Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2. Ryanodine (50 μM) was applied in the Ca^{2+}-free solution containing 10 mM caffeine for 10 min and the increase in Ca^{2+} subsequently induced by 10 mM caffeine or 10 μM NAd was abolished but that induced by 20 μM Ins(1,4,5)P_3 was only partly inhibited (50% of control). Ionomycin (1 μM) increased Ca^{2+} in ryanodine-treated skinned smooth muscle strips and completely inhibited the remaining increase in Ca^{2+} induced by the subsequent application of 20 μM Ins(1,4,5)P_3.

Effects of NAd on Ca^{2+} extrusion in intact smooth muscle strips

In the presence or absence of extracellular Ca^{2+}, the rate of decline of $[Ca^{2+}]_i$ as a function of $[Ca^{2+}]_i$ was studied in the presence of 128 mM K^+, 10 μM NAd or 10 mM caffeine (Fig. 5). The rate of decline of $[Ca^{2+}]_i$ was measured after the peak level of

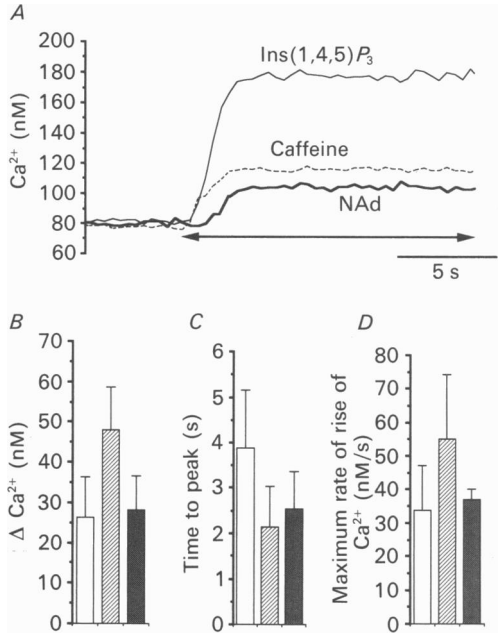

Fig. 4. Effects of 10 μM NAd, 20 μM Ins(1,4,5)P_3 or 10 mM caffeine on change in Ca^{2+} in β-escin-treated skinned smooth muscle strips of the rabbit mesenteric artery. After the strip was skinned, 0·3 μM Ca^{2+} buffered with 4 mM EGTA was applied for 2 min, Ca^{2+}-free solution containing 4 mM EGTA applied for 0·5 min and then 10 μM NAd, 10 mM caffeine or 20 μM Ins(1,4,5)P_3 applied for 1 min in Ca^{2+}-free solution containing 50 μM EGTA, 3 μM GTP and 2 μM Fura-2 (see also in Results). *A*, actual traces. 10 μM NAd, 20 μM Ins(1,4,5)P_3 or 10 mM caffeine was applied as indicated by ↔. *B*, the increase in Ca^{2+} induced by these agents. 'Δ Ca^{2+}' was calculated by the subtraction of the resting Ca^{2+} from the peak Ca^{2+}. *C*, the time required for Ca^{2+} to reach its peak after application of the stimuli. *D*, the maximum rate of rise of Ca^{2+} induced by 10 μM NAd (□), 20 μM Ins(1,4,5)P_3 (▨) or 10 mM caffeine (■). The rate of change of Ca^{2+} was calculated at 0·4 s intervals. Results shown are each the mean of five to nine observations with S.D. shown by a vertical bar.

$[Ca^{2+}]_i$ (indicated by arrows in Fig. 5) induced by the application of the individual agents and the rates were plotted against the corresponding $[Ca^{2+}]_i$. In Fig. 5*B* and *D*, the rate was calculated by averaging the values obtained over 1·2 s. In the presence of 128 mM K^+, the rate varied greatly. Since the rate of decline of $[Ca^{2+}]_i$ after the peak may depend on the balance between Ca^{2+}-influx and Ca^{2+}-removal mechanisms in the presence of the agonist, an activation of Ca^{2+} influx by 128 mM K^+ may have influenced the measurements of the rate of decay of $[Ca^{2+}]_i$ under the present experimental conditions.

In the presence of 10 mM caffeine or 10 μM NAd, the rate of decline of $[Ca^{2+}]_i$ depended on the $[Ca^{2+}]_i$ and the maximum rate of decline was obtained at around 350 nM $[Ca^{2+}]_i$ in the presence of either 10 μM NAd or 10 mM caffeine. The relationship between the rate of decline of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ in the presence of 10 μM

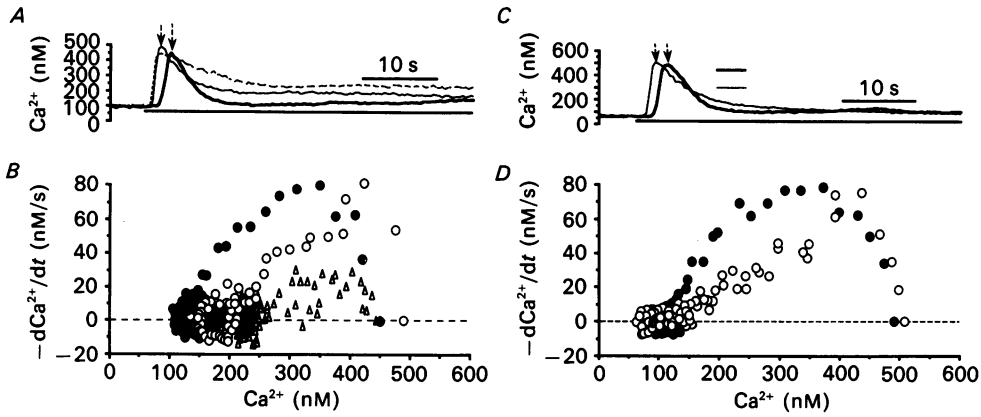

Fig. 5. The rate of decline of $[Ca^{2+}]_i$ as a function of $[Ca^{2+}]_i$ in the presence of 128 mM K^+, 10 μM NAd or 10 mM caffeine in a smooth muscle strip of the rabbit mesenteric artery. *A* and *C* show actual traces. In *A*, 128 mM K^+, 10 μM NAd or 10 mM caffeine was applied in Krebs solution (containing 2·6 mM Ca^{2+}). The rate of decline of $[Ca^{2+}]_i$ ($-d[Ca^{2+}]_i/dt$) was measured just after the peak $[Ca^{2+}]_i$ evoked by the individual stimuli (indicated by arrows). Dashed trace, high K^+ (128 mM); thicker continuous trace, NAd (10 μM); thinner continuous trace, caffeine (10 mM). *B*, the rate of decline as a function of $[Ca^{2+}]_i$ in the presence of high K^+ (128 mM, Δ), NAd (10 μM, ●) or caffeine (10 mM, ○). The results shown in *A* were used for this analysis. In order to reduce the noise, the rate of change in $[Ca^{2+}]_i$ was averaged for 1·2 s. In *C*, 10 μM NAd (thick trace) or 10 mM caffeine (thin trace) was applied 2 min after application of Ca^{2+}-free solution containing 2 mM EGTA. *D*, the rates of decline of $[Ca^{2+}]_i$ derived from the results shown in *C*. ●, 10 μM NAd; ○, 10 mM caffeine.

NAd or 10 mM caffeine was roughly the same whether in the presence or absence of extracellular Ca^{2+}.

When the relationships between the rate of decline of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ were compared for experiments with 10 μM NAd and 10 mM caffeine, the relationship was shifted to the left by the presence of the NAd whether in the presence or absence of extracellular Ca^{2+}. To minimize the participation of Ca^{2+} influx on the rate of decline of $[Ca^{2+}]_i$ in the presence of 10 μM NAd or 10 mM caffeine, the rates at 220 and 170 nM $[Ca^{2+}]_i$ were measured in Ca^{2+}-free solution containing 2 mM EGTA. The rates of decline of $[Ca^{2+}]_i$ at 220 and 170 nM $[Ca^{2+}]_i$ were respectively $-63{\cdot}0 \pm 6{\cdot}0$ and $-40{\cdot}3 \pm 3{\cdot}4$ nM/s in the presence of 10 μM NAd ($n = 10$) and $-17{\cdot}1 \pm 3{\cdot}6$ and $-6{\cdot}1 \pm 2{\cdot}5$ nM/s in the presence of 10 mM caffeine ($n = 6$). Thus, the removal of $[Ca^{2+}]_i$ is faster in the NAd-treated than the caffeine-treated strips. This could be interpreted as a speeding up of the 'normal rate' of Ca^{2+} removal by NAd or as a slowing by caffeine.

To study the roles of Ca^{2+} storage sites on the NAd-activated Ca^{2+} removal mechanisms, the effects of 10 μM NAd on the rate of decline of $[Ca^{2+}]_i$ were observed in the presence of 1 μM ionomycin in Ca^{2+}-free solution containing 2 mM EGTA (Fig.

6). After application of Ca^{2+}-free solution containing 2 mM EGTA for 2 min, 1 μM ionomycin was applied in the Ca^{2+}-free solution and this induced a transient increase in $[Ca^{2+}]_i$ from $84{\cdot}5 \pm 11{\cdot}2$ to $370 \pm 17{\cdot}5$ nM ($n = 5$), and followed by a gradual decay to the resting level. The $[Ca^{2+}]_i$ measured 10 min after application of 1 μM ionomycin

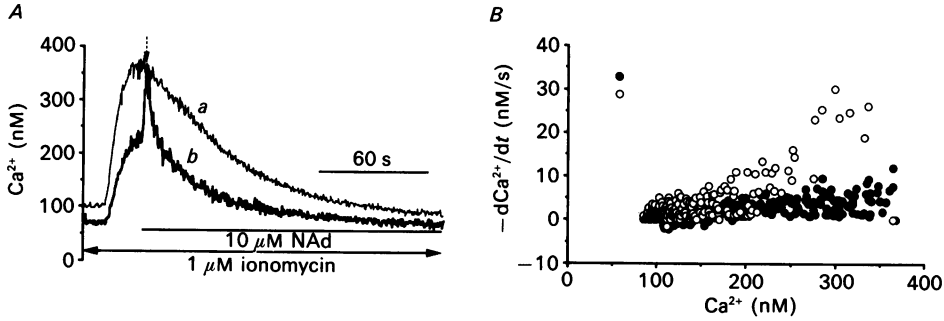

Fig. 6. Effects of 10 μM NAd on the rate of decline of $[Ca^{2+}]_i$ in the presence of 1 μM ionomycin in Ca^{2+}-free solution containing 2 mM EGTA. In *A*, 1 μM ionomycin was applied 2 min after application of Ca^{2+}-free solution containing 2 mM EGTA (*a*). NAd (10 μM) was applied near the peak $[Ca^{2+}]_i$ level evoked by 1 μM ionomycin (*b*). *a* and *b* were obtained from different smooth muscle strips. Strips were selected so that the peak $[Ca^{2+}]_i$ evoked by 1 μM ionomycin alone was identical to that evoked by ionomycin followed by NAd. The rate of decline of $[Ca^{2+}]_i$ ($-d[Ca^{2+}]_i/dt$) was measured just after the peak $[Ca^{2+}]_i$ evoked by ionomycin or ionomycin with NAd (indicated by arrow). *B*, the relationship between the rate of decline of $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ in the presence of 1 μM ionomycin with (○) or without (●) 10 μM NAd, where the rate of change of $[Ca^{2+}]_i$ was averaged for 1·2 s to reduce the noise. The results shown in *A* were used for this analysis.

in Ca^{2+}-free solution was $79{\cdot}1 \pm 11{\cdot}7$ nM ($n = 5$), and subsequent application of 10 μM NAd or 10 mM caffeine did not increase $[Ca^{2+}]_i$.

The rate of decline of $[Ca^{2+}]_i$ in the presence of ionomycin was so slow that it was difficult to analyse quantitatively the relationship between the rate and $[Ca^{2+}]_i$ (Fig. 6*B*). When 10 μM NAd was applied near the peak of the $[Ca^{2+}]_i$ evoked by 1 μM ionomycin, the rate of decline of $[Ca^{2+}]_i$ was accelerated (Fig. 6). When the maximum rates of decline of $[Ca^{2+}]_i$ induced by 10 μM NAd were compared in the presence or absence of 1 μM ionomycin, these were $-27{\cdot}2 \pm 5{\cdot}3$ nM Ca^{2+}/s ($n = 4$) and $-83{\cdot}2 \pm 15$ nM Ca^{2+}/s ($n = 6$), respectively. The rates of decline of $[Ca^{2+}]_i$ observed for $[Ca^{2+}]_i$ over the range 150–300 nM were also smaller in the presence of ionomycin than in its absence (Fig. 6*B*).

Effects of NAd on the relationship between $[Ca^{2+}]_i$ and tension in intact smooth muscle strips

Figure 7 shows the relationship between $[Ca^{2+}]_i$ and tension in the presence of 128 mM K^+ or 10 μM NAd in smooth muscle strips of the rabbit mesenteric artery. In Fig. 7*A*, the relationship between $[Ca^{2+}]_i$ and tension is plotted as a function of time after application of either stimulus. Since each stimulant was injected over 1 s in the present experiments, the initial phase of the tension response (within 1 s) was impossible to measure and so the measurement of tension was begun from just after the injection. The plotted points shown in Fig. 7*A* indicate the tonic responses

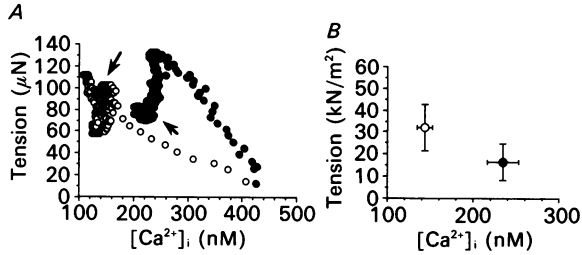

Fig. 7. The relationship between $[Ca^{2+}]_i$ and tension as a function of time in the presence of 128 mM K^+ or 10 μM NAd in smooth muscle strips of the rabbit mesenteric artery. High K^+ (128 mM) or NAd (10 μM) was applied in Krebs solution (containing 2·6 mM Ca^{2+}). *A*, the tension–$[Ca^{2+}]_i$ relationships are shown as a function of time in the presence of 128 mM K^+ or 10 μM NAd. After application of either stimulus, $[Ca^{2+}]_i$ immediately increased without an increase in tension, and $[Ca^{2+}]_i$ then decayed as tension developed. The arrows indicate the tonic phase of the response in the presence of 128 mM K^+ (●) or 10 μM NAd (○). *B*, the relationship between $[Ca^{2+}]_i$ and tension during the tonic phase in the presence of 128 mM K^+ (●) or 10 μM NAd (○). The relationships were obtained 2 min after application of either stimulus. Results shown are each the mean of five to seven observations with S.D. shown by a vertical bar.

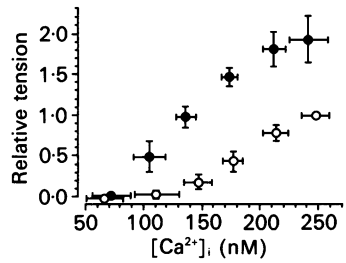

Fig. 8. Effects of 10 μM NAd on $[Ca^{2+}]_i$–tension relationship in ionomycin-treated smooth muscle strips of the rabbit mesenteric artery. The muscle strips were treated with 1 μM ionomycin for 20 min in Krebs solution. Ca^{2+}-free solution containing 2 mM EGTA with 5·9 mM K^+ was then applied for 1 min followed by the application of Ca^{2+}-free solution containing 2 mM EGTA with 128 mM K^+ in the presence or absence of 10 μM NAd. Finally, solutions containing various concentrations of Ca^{2+} from 0·08 to 2·6 mM with 128 mM K^+ were cumulatively applied from low to high in the absence (control, ○) or presence (●) of 10 μM NAd. The maximum tension induced by 2·6 mM Ca^{2+} in the absence of 10 μM NAd was normalized as a relative tension of 1·0. Each symbol represents the mean of seven observations with S.D. shown by a vertical bar.

induced by 128 mM K^+ and 10 μM NAd (shown by the arrows). Although the magnitude of the tonic tension induced by the two stimuli was similar, the plotted points are present at lower values of $[Ca^{2+}]_i$ in the presence of 10 μM NAd than in 128 mM K^+ (Fig. 7*A*). The relationship between $[Ca^{2+}]_i$ and tension in the tonic phase induced by 128 mM K^+ or 10 μM NAd was observed 2 min after application of the stimulus. As shown in Fig. 7*B*, 10 μM NAd produced a larger tension with a smaller increase in $[Ca^{2+}]_i$ than did 128 mM K^+ ($n = 5$).

The effect of 10 μM NAd on the relationship between $[Ca^{2+}]_i$ and tension was studied in Ca^{2+}-free solution containing 128 mM K^+ and 2 mM EGTA (Fig. 8). To increase $[Ca^{2+}]_i$ and tension, various concentrations of Ca^{2+} from 0·08 to 2·6 mM were

cumulatively applied following application of ionomycin (1 μM) to the muscle strips for 20 min to deplete the stored Ca^{2+}.

Noradrenaline (10 μM) increased the amplitude of contraction without altering the corresponding level of $[Ca^{2+}]_i$ induced by any of the various concentrations of Ca^{2+} (0·08–2·6 mM) in Ca^{2+}-free solution containing 128 mM K^+. NAd (10 μM) shifted the $[Ca^{2+}]_i$–tension relationship to the left and increased the maximum amplitude of contraction induced by 2·6 mM Ca^{2+}. The half-maximum amplitude of contraction was obtained at $189{\cdot}0 \pm 16{\cdot}5$ and $134{\cdot}4 \pm 13{\cdot}9$ nM $[Ca^{2+}]_i$ in the absence and presence of 10 μM NAd, respectively ($P < 0{\cdot}05$, $n = 5$). These effects of NAd were inhibited by 1 μM prazosin.

DISCUSSION

In an earlier paper on the smooth muscle of the rabbit mesenteric artery, we reported (1) that NAd binds to α-receptors and synthesizes Ins(1,4,5)P_3 in intact smooth muscle and (2) that Ins(1,4,5)P_3 produces contraction in Ca^{2+}-free solution containing a low concentration of EGTA (0·05–0·1 mM) after brief application of Ca^{2+} in saponin-treated skinned smooth muscle (Hashimoto *et al.* 1986). In α-toxin- or β-escin-treated skinned smooth muscle strips of the rabbit main pulmonary artery, phenylephrine and Ins(1,4,5)P_3 each increased tension in Ca^{2+}-free solution containing 50 μM EGTA after application of Ca^{2+}, and these effects were inhibited by heparin, an inhibitor of the Ins(1,4,5)P_3 receptor (Kitazawa *et al.* 1989; Kobayashi *et al.* 1989). In the present experiments, NAd increased the concentration of Ins(1,4,5)P_3 within 5 s in intact smooth muscle strips of the rabbit mesenteric artery and, in β-escin-treated skinned smooth muscle, Ins(1,4,5)P_3 and NAd each increased Ca^{2+} in Ca^{2+}-free solution containing 50 μM EGTA and 2 μM Fura-2 following brief application of 0·3 μM Ca^{2+}. These results suggest that Ins(1,4,5)P_3 synthesized by NAd may be a second messenger for Ca^{2+} release induced by this agonist in vascular smooth muscle.

The time required for $[Ca^{2+}]_i$ to reach its maximum rate of rise after application of 10 μM NAd in β-escin-treated skinned smooth muscle was almost the same as in intact smooth muscle ($2{\cdot}8 \pm 0{\cdot}5$ s in the skinned smooth muscle and $3{\cdot}7 \pm 0{\cdot}7$ s in intact smooth muscle), suggesting that NAd releases Ca^{2+} in a similar manner in the two conditions. In β-escin-treated skinned smooth muscle strips, the time for Ca^{2+} to reach its maximum rate of rise or its peak was slightly longer in the presence of 10 μM NAd than with 20 μM Ins(1,4,5)P_3 or 10 mM caffeine (time to peak: 3·9 s in 10 μM NAd, 2·1 s in Ins(1,4,5)P_3 and 2·5 s in caffeine; time to reach maximum rate of rise: 2·1 s in 10 μM NAd, 1·0 s in Ins(1,4,5)P_3 and 1·0 s in caffeine). Further, the time lag was always observed for NAd-induced increases in $[Ca^{2+}]_i$ whether in intact or β-escin-skinned smooth muscle strips.

Somlyo, Walker, Goldman, Trentham & Kobayashi (1988) found that using photolabile but biologically inactive (termed 'caged') phenylephrine or caged Ins(1,4,5)P_3, the lag phase preceding force development after flash photolysis was 0·4 s for Ins(1,4,5)P_3 in saponin-treated skinned smooth muscle and 1·8 s for phenylephrine in the membrane-depolarized, intact smooth muscle of the guinea-pig portal vein at 20 °C. They suggested that the long delay (1·5 s at 22 °C) following activation of α_1-adrenergic receptors through the photolysis of caged phenylephrine is consistent

with the hypothesis that activation of phospholipase C is the major mechanism in α_1-adrenergic Ca^{2+} release (Somlyo & Somlyo, 1990). These results suggest that the time lag before the initiation of the NAd-induced increase in $[Ca^{2+}]_i$ in our experiments may relate to the processes involved from α-receptor binding to the production of Ins(1,4,5)P_3.

Following application of 10 mM caffeine in Ca^{2+}-free solution, 10 μM NAd failed to increase $[Ca^{2+}]_i$ in intact smooth muscle strips of the rabbit mesenteric artery. Ryanodine inhibited the increase in $[Ca^{2+}]_i$ induced by 10 μM NAd or 10 mM caffeine in intact and skinned smooth muscle strips. Although 20 μM Ins(1,4,5)P_3 still increased Ca^{2+} in ryanodine-treated skinned smooth muscle strips (as also reported in guinea-pig taenia caeci; Iino, 1990), these results suggest that NAd, at this concentration, releases Ca^{2+} from the ryanodine-sensitive Ca^{2+} storage sites in smooth muscle of the rabbit mesenteric artery.

NAd increases the rate of decline of $[Ca^{2+}]_i$

In vascular smooth muscle, agonists increase $[Ca^{2+}]_i$ not only by release of $[Ca^{2+}]_i$ but also by activation of Ca^{2+} influx (Kuriyama *et al.* 1982; Leitzen & van Breemen, 1984). Using the Ca^{2+}-sensitive photoprotein aequorin, Morgan & Morgan (1984) reported that phenylephrine only transiently increased $[Ca^{2+}]_i$ in the ferret portal vein. In Fura-2-loaded single smooth muscle cells of the porcine coronary artery, acetylcholine only transiently increased $[Ca^{2+}]_i$ and this was followed by a gradual decay within 1 min (Itoh, Kubota & Kuriyama, 1988). It has recently been suggested that some agonists activate the Ca^{2+} extrusion mechanism on the plasma membrane in various types of cells (Carafoli, 1987). These results raise the possibility that agonists may accelerate the Ca_i^{2+} removal mechanisms and thus overcome the effect of Ca^{2+} influx induced by the agonists themselves in vascular smooth muscle cells, with the result that the cells can maintain the steady-state $[Ca^{2+}]_i$ at a low level even in the presence of the agonist.

Following receptor activation, several processes that can restore $[Ca^{2+}]_i$ to the resting level might be activated. These are as follows: (1) inactivation of the receptor-mediated transduction processes, (2) re-sequestration of Ca^{2+} into the cellular storage sites and (3) extrusion of Ca^{2+} to the extracellular space. These processes may be activated by the receptor-mediated intracellular 2nd messengers, for instance Ca^{2+}-calmodulin, cyclic AMP or cyclic GMP and diacylglycerol (Carafoli, 1987; Rink & Sage, 1987).

In the present experiments, NAd accelerated the rate of decline of $[Ca^{2+}]_i$ more than did caffeine in low $[Ca^{2+}]_i$ conditions (range 150–350 nM) whether in the presence or absence of extracellular Ca^{2+}. The restoration of $[Ca^{2+}]_i$ following application of 1 μM ionomycin was slow at any $[Ca^{2+}]_i$, but was stimulated by 10 μM NAd. The maximum rate of decline of $[Ca^{2+}]_i$ induced by 10 μM NAd in ionomycin-treated muscle strips was almost one-third that observed in muscle strips not treated with ionomycin. Since ionomycin releases any Ca^{2+} pumped back into the cellular Ca^{2+} storage sites (Rink & Sage, 1987), the removal of Ca^{2+} from the cytosol in Ca^{2+}-free solution is supposed to be achieved by Ca^{2+} extrusion to the extracellular space. These results suggest that NAd activates not only Ca^{2+} sequestration to the storage sites, but also Ca^{2+}-extrusion mechanisms in smooth muscle of the rabbit mesenteric

artery. Since 0·3 μM propranolol had no effect on the rate of decline of $[Ca^{2+}]_i$ in the presence of NAd, any role of cyclic-AMP-dependent mechanisms in the NAd-induced acceleration of the decline of $[Ca^{2+}]_i$ may be ruled out.

In human platelets, Rink & Sage (1987) reported that the restoration of $[Ca^{2+}]_i$ following application of ionomycin was stimulated by thrombin or by phorbol myristate acetate (PMA, an activator of protein kinase C), suggesting that protein kinase C may have a role in this thrombin-stimulated Ca^{2+}-extrusion mechanism. Almost the same stimulating effect of PMA on $^{45}Ca^{2+}$ efflux was reported in cultured vascular smooth muscle from the rat thoracic aorta (Furukawa, Tawada & Shigekawa, 1989). In the preliminary experiments using smooth muscle strips of the rabbit mesenteric artery, staurosporine (0·1 and 1 nM), an inhibitor of protein kinase C (Tamaoki, Nomoto, Takahashi, Kato, Morimoto & Tomita, 1986), inhibited in a concentration-dependent manner the extent of the increase in $[Ca^{2+}]_i$ and the rate of decline of $[Ca^{2+}]_i$ in the presence of NAd in Ca^{2+}-free solution. By contrast, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride (H-7, 1–3 μM), another inhibitor of protein kinase C (Hidaka, Inagaki, Kawamoto & Sasaki, 1984), induced a concentration-dependent inhibition of the extent of the increase in $[Ca^{2+}]_i$ but had no effect on the rate of decline of $[Ca^{2+}]_i$ in the presence of NAd in Ca^{2+}-free solution. Thus, at present, the possible role of protein kinase C in the NAd-induced acceleration of the rate of decline of $[Ca^{2+}]_i$ is unclear.

NAd increases the sensitivity of the contractile proteins to Ca^{2+} in intact smooth muscle

The tension/Ca^{2+} ratio is higher on agonist-induced, than on high-K^+-induced stimulation (Morgan & Morgan, 1984). In the present experiments, 10 μM NAd induced the development of more tonic tension than did 128 mM K^+ with a smaller increase in $[Ca^{2+}]_i$ level. When the $[Ca^{2+}]_i$–tension relationship was studied using cumulative application of Ca^{2+} (0·08–2·6 mM) in ionomycin- and 128 mM K^+-treated smooth muscle strips, NAd shifted the relationship to the left without altering the corresponding levels of $[Ca^{2+}]_i$ and also increased the maximum amplitude of contraction. Under these experimental conditions, any contribution by released Ca^{2+} from the storage sites may be negligible with respect to the change in relationship, since ionomycin was present. These results suggest that, with the membrane depolarized by 128 mM K^+, NAd enhances tension as a result of an increase in the sensitivity of contractile proteins to Ca^{2+}, and not by activation of Ca^{2+} influx.

In α-toxin-treated skinned smooth muscle, muscarinic and α-adrenergic agonists (in the presence of guanosine triphosphate (GTP) or guanosine 5′-*O*-(γ-thio)-triphosphate (GTPγS)) and GTPγS itself can increase the contractile response to a given submaximal level of Ca^{2+} in some vascular tissues (Nishimura *et al.* 1988; Kitazawa *et al.* 1989; Kobayashi *et al.* 1989). In β-escin- and ionomycin-treated skinned smooth muscle strips of the rabbit mesenteric artery, NAd (10 μM) with GTP (3 μM) shifted the relationship to the left and enhanced the maximum tension induced by 10 μM Ca^{2+} (Itoh *et al.* 1991). Since guanosine 5′-*O*-(β-thio)diphosphate (GDPβS) or neomycin (an inhibitor of phospholipase C) inhibited the sensitizing effects of the agonists (Kitazawa *et al.* 1989), it can be suggested that the sensitization is mediated by G-proteins. In a recent review, Somlyo & Somlyo (1990) reported that the sensitization of α-toxin-treated skinned smooth muscle to Ca^{2+} by

photolysis of caged GTPγS involves a very long time delay (10–15 s) in the rabbit portal vein. If this is the case for NAd-induced contraction in smooth muscle of the rabbit mesenteric artery, mechanisms other than a change in $[Ca^{2+}]_i$ may operate in the later phase of the NAd-induced contraction to enhance the tension induced by this agonist at lower levels of $[Ca^{2+}]_i$.

In conclusion, in smooth muscle of the rabbit mesenteric artery NAd releases Ca^{2+} from the ryanodine-sensitive Ca^{2+} storage sites through the action of synthesized Ins(1,4,5)P_3. NAd promotes the process which restores $[Ca^{2+}]_i$ to the resting level through activation of both the sequestration of Ca^{2+} and the extrusion of Ca^{2+}. NAd also increases the sensitivity of contractile proteins to Ca^{2+} and thus induces development of more tension at low $[Ca^{2+}]_i$ levels.

We thank Drs R. J. Timms and K. Creed for the language editing. This work was partly supported by a Grant-In-Aid from the Ministry of Education of Japan and by the Japan Heart Foundation.

REFERENCES

BECKER, P. L., SINGER, J. J., WALSH, J. V. & FAY, F. S. (1989). Regulation of calcium concentration in voltage-clamped smooth muscle cells. *Science* **244**, 211–214.

CARAFOLI, E. (1987). Intracellular calcium homeostasis. *Annual Review of Biochemistry* **56**, 395–433.

EGGERMONT, J. A., VROLIX, M., RAEYMAEKERS, L., WUYTACK, F. & CASTEELS, R. (1988). Ca^{2+}-transport ATPases of vascular smooth muscle. *Circulation Research* **62**, 266–278.

FLEISCHER, S., OGUNBUNMI, E. M., DIXON, M. C. & FLEER, E. A. M. (1985). Localization of Ca^{2+} release channels with ryanodine in junctional terminal cisternae of sarcoplasmic reticulum of fast skeletal muscle. *Proceedings of the National Academy of Sciences of the USA* **82**, 7256–7259.

FURUKAWA, K., TAWADA, Y. & SHIGEKAWA, M. (1989). Protein kinase C activation stimulates plasma membrane Ca^{2+} pump in cultured vascular smooth muscle cells. *Journal of Biological Chemistry* **264**, 4844–4849.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440–3450.

HASHIMOTO, T., HIRATA, M., ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inositol 1,4,5-trisphosphate activates pharmacomechanical coupling in smooth muscle of the rabbit mesenteric artery. *Journal of Physiology* **370**, 605–618.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinoline-sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinases and protein kinase C. *Biochemistry* **23**, 5036–5041.

HIMPENS, B., MATTHIJS, G. & SOMLYO, A. P. (1989). Desensitization to cytoplasmic Ca^{2+} and Ca^{2+} sensitivities of guinea-pig ileum and rabbit pulmonary artery smooth muscle. *Journal of Physiology* **413**, 489–503.

IINO, M. (1990). Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. *Journal of General Physiology* **95**, 1103–1122.

ITOH, T., KANMURA, Y. & KURIYAMA, H. (1985). A23187 increases calcium permeability of store sites more than of surface membranes in the rabbit mesenteric artery. *Journal of Physiology* **359**, 467–484.

ITOH, T., KANMURA, Y. & KURIYAMA, H. (1986). Inorganic phosphate regulates the contraction–relaxation cycle in skinned muscles of the rabbit mesenteric artery. *Journal of Physiology* **376**, 231–252.

ITOH, T., KUBOTA, Y. & KURIYAMA, H. (1988). Effects of a phorbol ester on acetylcholine-induced Ca^{2+} mobilization and contraction in the porcine coronary artery. *Journal of Physiology* **397**, 401–419.

ITOH, T., KURIYAMA, H. & SUZUKI, H. (1983). Differences and similarities in noradrenaline- and caffeine-induced mechanical responses in the rabbit mesenteric artery. *Journal of Physiology* **337**, 609–629.

ITOH, T., SUZUKI, S. & KURIYAMA, H. (1991). Effects of pinacidil on contractile proteins in high K^+-treated intact, and in β-escin-treated skinned smooth muscle of the rabbit mesenteric artery. *British Journal of Pharmacology* **103**, 1697–1702.

KITAZAWA, T., KOBAYASHI, S., HORIUTI, K., SOMLYO, A. V. & SOMLYO, A. P. (1989). Receptor-coupled, permeabilized smooth muscle. *Journal of Biological Chemistry* **264**, 5339–5342.

KOBAYASHI, S., KITAZAWA, T., SOMLYO, A. V. & SOMLYO, A. P. (1989). Cytosolic heparin inhibits muscarinic and α-adrenergic Ca^{2+} release in smooth muscle. *Journal of Biological Chemistry* **264**, 17997–18004.

KURIYAMA, H., ITO, Y., SUZUKI, H., KITAMURA, K. & ITOH, T. (1982). Factors modifying contraction–relaxation cycle in vascular smooth muscles. *American Journal of Physiology* **243**, H641–662.

LEITZEN, P. A. A. & VAN BREEMEN, C. (1984). The effects of caffeine on the noradrenaline-sensitive calcium store in rabbit aorta. *Journal of Physiology* **357**, 327–339.

MORGAN, J. P. & MORGAN, K. G. (1984). Alteration of cytoplasmic ionized calcium levels in smooth muscle by vasodilators in the ferret. *Journal of Physiology* **357**, 539–551.

NISHIMURA, J., KOLBER, M. & VAN BREEMEN, C. (1988). Norepinephrine and GTP-γ-S increase myofilament Ca^{2+} sensitivity in α-toxin permeabilized arterial smooth muscle. *Biochemical and Biophysical Research Communications* **157**, 677–683.

POENIE, M., ALDERTON, J., STEINHART, R. A. & TSIEN, R. Y. (1986). Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science* **233**, 886–889.

RINK, T. J. & SAGE, S. O. (1987). Stimulated calcium efflux from Fura-2-loaded human platelets. *Journal of Physiology* **393**, 513–524.

ROUSSEAU, E., SMITH, J. S. & MEISSNER, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channel. *American Journal of Physiology* **253**, C364–368.

SOMLYO, A. P. & SOMLYO, A. V. (1990). Flash photolysis studies of excitation–contraction coupling, regulation, and contraction in smooth muscle. *Annual Review of Physiology* **52**, 857–874.

SOMLYO, A. P., WALKER, J. W., GOLDMAN, Y. E., TRENTHAM, D. R. & KOBAYASHI, S. (1988). Inositol trisphosphate, calcium and muscle contraction. *Philosophical Transactions of the Royal Society* B **320**, 399–414.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. *Biochemical and Biophysical Research Communications* **135**, 397–402.