British Journal of Pharmacology (1997) 121, 1155–1163

Possible mechanisms underlying the midazolam-induced relaxation of the noradrenaline-contraction in rabbit mesenteric resistance artery

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1 The mechanisms underlying the midazolam-induced relaxation of the noradrenaline (NA)-contraction were studied by measuring membrane potential, isometric force and intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in endothelium-denuded muscle strips from the rabbit mesenteric resistance artery. The actions of midazolam were compared with those of nicardipine, an L-type Ca^{2+}-channel blocker.

2 Midazolam (30 and 100 μM) did not modify either the resting membrane potential or the membrane depolarization induced by 10 μM NA.

3 NA (10 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. Midazolam (10–100 μM) did not modify the resting $[Ca^{2+}]_i$, but attenuated the NA-induced phasic and tonic increases in $[Ca^{2+}]_i$ and force, in a concentration-dependent manner. In contrast, nicardipine (0.3 μM) attenuated the NA-induced tonic, but not phasic, increases in $[Ca^{2+}]_i$ and force.

4 In Ca^{2+}-free solution containing 2 mM EGTA, NA (10 μM) transiently increased $[Ca^{2+}]_i$ and force. Midazolam (10–100 μM), but not nicardipine (0.3 μM), attenuated this NA-induced increase in $[Ca^{2+}]_i$ and force, in a concentration-dependent manner. However, midazolam (10 and 30 μM), had no effect on the increases in $[Ca^{2+}]_i$ and force induced by 10 mM caffeine.

5 In ryanodine-treated strips, which have functionally lost the NA-sensitive Ca^{2+}- storage sites, NA slowly increased $[Ca^{2+}]_i$ and force. Nicardipine (0.3 μM) did not modify the resting $[Ca^{2+}]_i$ but partly attenuated the NA-induced increases in $[Ca^{2+}]_i$ and force. In the presence of nicardipine, midazolam (100 μM) lowered the resting $[Ca^{2+}]_i$ and further attenuated the remaining NA-induced increases in $[Ca^{2+}]_i$ and force.

6 The $[Ca^{2+}]_i$-force relationship was obtained in ryanodine-treated strips by the application of ascending concentrations of Ca^{2+} (0.16–2.6 mM) in Ca^{2+}-free solution containing 100 mM K^+. NA (10 μM) shifted the $[Ca^{2+}]_i$-force relationship to the left and enhanced the maximum Ca^{2+}-induced force. Under these conditions, whether in the presence or absence of 10 μM NA, midazolam (10 and 30 μM) attenuated the increases in $[Ca^{2+}]_i$ and force induced by Ca^{2+} without changing the $[Ca^{2+}]_i$-force relationship.

7 It was concluded that, in smooth muscle of the rabbit mesenteric resistance artery, midazolam inhibits the NA-induced contraction through its inhibitory action on NA-induced Ca^{2+} mobilization. Midazolam attenuates NA-induced Ca^{2+} influx via its inhibition of both nicardipine-sensitive and -insensitive pathways. Furthermore, midazolam attenuates the NA-induced release of Ca^{2+} from the storage sites. This effect contributes to the midazolam-induced inhibition of the NA-induced phasic contraction.

Keywords: Midazolam; noradrenaline-induced Ca^{2+} mobilization; myofilament Ca^{2+} sensitivity; agonist-induced Ca^{2+} release; vascular smooth muscle

Introduction

Midazolam, a benzodiazepine agonist, has been used as an anti-anxiety drug, for producing hypnosis and for the induction of general anaesthesia. Midazolam induces hypotension both when used in combination with narcotics (West *et al*., 1987) and during cardiopulmonary bypass (McNulty *et al*., 1994). Since the hypotension is accompanied by a decrease in peripheral vascular resistance (West *et al*., 1987; Windsor *et al*., 1988; McNulty *et al*., 1994), it is thought the midazolam-induced hypotension is probably due to its direct vasodilating action on peripheral resistance arteries.

It is generally thought that the contraction-relaxation cycle in vascular smooth muscle depends largely on the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). An increase in $[Ca^{2+}]_i$ in smooth muscle cells can occur through activation not only of Ca^{2+} influx, but of Ca^{2+} release from the intracellular stores. It has been shown that in many arterial smooth muscle cells, Ca^{2+}-mobilizing agonists activate both the dihydropyridine-sensitive and insensitive pathways (Kuriyama *et al*., 1995). These agonists also activate the hydrolysis of phosphatidylinositol 4,5-bisphosphate and thus produce inositol 1,4,5-trisphosphate ($InsP_3$), which causes a release of Ca^{2+} from the stores. In addition, it has been suggested that such agonists enhance the contraction that occurs at a given concentration of $[Ca^{2+}]_i$ (an agonist-induced increase in myofilament Ca^{2+}-sensitivity), thus causing a maintained contraction at a relatively low $[Ca^{2+}]_i$ (Nishimura *et al*., 1988; Fujiwara *et al*., 1989; Kitazawa *et al*., 1989; Itoh *et al*., 1992a,b).

In the rat aorta, it has been shown that midazolam inhibits voltage-operated Ca^{2+} channels and attenuates agonist-induced contractions (French *et al*., 1989; Chang *et al*., 1994).

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However, it remains unclear whether the vasodilating effect of midazolam that is exerted via agonist-induced Ca^{2+} influxes is simply mediated through an inhibition of a dihyrdropyridine-sensitive pathway. We recently found that the membrane hyperpolarization induced by K_{ATP}-channel openers relaxes smooth muscle both by inhibition of the activation of voltage-dependent Ca^{2+} channels and also by attenuation of NA-induced inositol 1,4,5-trisphosphate ($InsP_3$) synthesis (Itoh *et al*., 1992b, Watanabe *et al*., 1996). Carlen *et al*., (1983) showed that midazolam exerts a hyperpolarizing effect on the plasma membrane of cells in the central nervous system in addition to its effect on the γ-aminobutyrate (GABA)-receptor complex. However, in resistance arterial smooth muscle cells, it is unclear (i) whether midazolam hyperpolarizes the membrane, (ii) whether this agent affects agonist-induced Ca^{2+} release from the storage sites, and (iii) whether midazolam affects the agonist-induced increase in myofilament Ca^{2+}-sensitivity. Thus, the mechanism underlying the vasorelaxation induced by midazolam in peripheral resistance arteries remains uncertain.

The present study was therefore performed to clarify the mechanism underlying the vasorelaxing action of midazolam on the noradrenaline (NA)-induced contraction in a resistance artery. To do this, we measured the membrane potential, $[Ca^{2+}]_i$ and isometric force in fine endothelium-denuded strips from a resistance artery of the rabbit mesentery. To assess the effect of midazolam on NA-induced Ca^{2+} release, we observed its effect on the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution. To assess the effect of midazolam on NA-induced Ca^{2+} influx, its effect was observed on the NA-induced increase in $[Ca^{2+}]_i$ in ryanodine-treated muscle strips (which have functionally lost the NA-sensitive Ca^{2+} storage sites) (Itoh *et al*., 1992a; Watanabe *et al*., 1996). To study the effect of midazolam on the NA-induced increase in myofilament Ca^{2+}-sensitivity, the effect of midazolam was examined on the $[Ca^{2+}]_i$-force relationship obtained in the presence of NA in ryanodine-treated muscle strips.

Methods

Male Japan White albino rabbits (supplied from Kitayama Labes Co. Ltd, Japan), weighing 1.9–2.5 kg, were anaesthetized by injection of pentobarbitone sodium (40 mg kg^{-1}, i.v.) and then exsanguinated. The protocols used conformed with guidelines on the conduct of animal experiments issued by Nagoya City University Medical School and by the Japanese government (Law [no. 105]; Notification [no. 6]), and were approved by The Committee on the Ethics of Animal Experiments in Nagoya City University Medical School. The third branch of the mesenteric artery distributing to the region of the ileum (diameter approximately 70–100 μm) was excised immediately and cleaned by removal of connective tissue in Krebs solution under a binocular microscope at room temperature. After the artery had been cut open along its longitudinal axis with small scissors, the endothelium of the strip was carefully removed by gentle rubbing of the internal surface of the vessel using small pieces of razor blade, as described previously (Itoh *et al*.,1992a,b). Satisfactory ablation of the endothelium was pharmacologically verified by the absence of a relaxing effect of 3–10 μM acetylcholine during a NA-contraction.

Membrane potential measurements

A strip of the artery was placed in a chamber of 0.5 ml volume on an invert-microscope (Diaphoto TMD, Nikon, Japan) and superfused with Krebs solution. Glass microelectrodes were made from borosilicate glass tubing (o.d. 1.2 mm with a glass filament inside, Hilgenberg, Germany) and filled with 1 M KCl. The resistance of the electrodes was 120–180 MΩ. The electrode was inserted into a smooth muscle cell from the luminal side. Membrane potentials were recorded with an Axoclamp-2B amplifier (Axon Instruments) and displayed on a cathode-ray oscilloscope (Hitachi). The data was stored at an acquisition rate of 200 Hz by an AxoScope 1.1/Digidata 1200 data acquisition system (Axon Instruments) on an IBM-compatible PC. To observe the effect of midazolam on the membrane potential, this agent was applied for 10 min before and throughout the application of 10 μM NA.

$[Ca^{2+}]_i$ and force measurement

To enable simultaneous recording of isometric force and $[Ca^{2+}]_i$, fine circularly-cut strips (0.3–0.5 mm long, 0.04–0.05 mm wide, 0.02–0.03 mm thick) were prepared as described previously (Itoh *et al*., 1992a,b). The strips were transferred to chambers of 0.3 ml volume and mounted horizontally on an invert-microscope (Diaphoto TMD with special optics for epifluorescence, Nikon). The resting force was adjusted to obtain a maximum contraction in Krebs solution containing 128 mM K^+.

To enable loading of Fura 2 into smooth muscle cells of the strip, 1.2 μM acetoxymethyl ester of Fura 2 (Fura 2-AM) was applied for 1.5 h in Krebs solution at room temperature. After this period, the solution containing Fura 2-AM was washed out with Krebs solution for 1 h to ensure sufficient 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 background fluorescence (0.04 mm square). The Fura 2 fluorescence emission at 510 nm (by use of an interference filter centred at 510 nm and a full width at half-transmission of 20 nm) was passed through the lens (20 times CF Fluor objective lens, Nikon) and collected in a photomultiplier tube (R 928, side-on type, Hamamatsu Photonics, Japan) via a dichroic mirror (DM-400, Nikon) which was substituted for the photochanger in a Nikon Diaphoto-TMD microscope. Two alternative excitation wavelengths, 340 nm and 380 nm (each slit 5 nm), were applied by a spectro-fluorimeter (CA 200 DP, Japan Spectroscopic Co. Ltd., Tokyo, Japan) and the data were analysed by software developed in our laboratory. The ratio of the Fura 2 fluorescence intensities excited by 340 or 380 nm was calculated after subtraction of the background fluorescence and the $[Ca^{2+}]_i$ was calculated by use of the formula described by Grynkiewicz *et al*. (1985) and an *in vitro* calibration procedure (Poenie *et al*., 1986). The ratio of maximum (F_{max}) to minimum (F_{min}) fluorescence 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; Itoh *et al*., 1992a,b). The K_d value for Fura 2 was estimated to be 200 nM (Itoh *et al*., 1992a). This method of calculating $[Ca^{2+}]_i$ may not be accurate if Fura 2 binds to some proteins in the cell (Konishi *et al*., 1988).

NA (10 μM), high K^+ or caffeine (10 mM) was applied for 2 min at 20 min intervals in Krebs solution, so that reproducible responses could be obtained. Midazolam (10–100 μM) or nicardipine (0.3 μM) was applied for 10 min before and throughout the applications of NA, high K^+ or caffeine.

To observe the effect of midazolam on the NA-induced release of Ca^{2+} from the storage sites, experiments were carried out in the Ca^{2+}-free solution containing 2 mM ethyleneglycol-bis-(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) with 5.9 mM K^+. After 2 min in Ca^{2+}-free solution, the strips were stimulated by 10 μM NA for 2 min and then brought back to Ca^{2+}-containing Krebs solution (Ca^{2+} = 2.6 mM) for 20 min. Midazolam (10–100 μM) or nicardipine (0.3 μM) was applied for 10 min in Krebs solution and was present in the Ca^{2+}-free solution and during the application of NA. In another set of experiments, 10 μM NA and 10 mM caffeine were applied successively (with an interval of 5 min) in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. In these experiments, after 2 min removal of Ca^{2+} by application of the Ca^{2+}-free solution, 10 μM NA was first applied for 2 min, followed by a 5 min washout of NA. Caffeine (10 mM) was then applied in the Ca^{2+}-free solution and, finally, the strip

was brought back to Krebs solution for 20 min. Midazolam (10 and 30 μM) was pretreated for 10 min before, and was present during, the application of NA. It was then washed out (for 5 min) and was not present during the subsequent application of caffeine.

In some experiments, Ca^{2+} storage sites in the smooth muscle cells were functionally removed by the application of ryanodine (Fleischer *et al*., 1985; Itoh *et al*., 1992b). After the response induced by 10 μM NA had been recorded, ryanodine (50 μM) together with 10 mM caffeine was applied for 5 min in Krebs solution followed by a 10 min application of Krebs solution containing 10 μM ryanodine alone; 10 μM NA was then again applied in the presence of ryanodine. Under these conditions, 10 μM NA was applied for 3 min at 30 min intervals in the presence of 10 μM ryanodine to obtain reproducible responses. Nicardipine (0.3 μM) was first applied 10 min before and was present during the application of NA. After 30 min washout of NA by Krebs solution containing nicardipine with ryanodine, 100 μM midazolam was then applied for 10 min before and throughout the application of NA in the presence of nicardipine with ryanodine.

The effect of midazolam on the $[Ca^{2+}]_i$-force relationship was studied in the presence and absence of 10 μM NA in ryanodine-treated muscle strips. Ryanodine-treatment was administered to the muscle strips as described above. Following the ryanodine-treatment, Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ was applied for 1 min, and then Ca^{2+}-free solution containing 100 mM K^+ with 2 mM EGTA was applied for 1 min in the presence or absence of 10 μM NA. At this point, various concentrations of Ca^{2+} (0.16–2.6 mM) were cumulatively applied for 2 min in an ascending order together with 100 mM K^+ in the presence and absence of 10 μM NA. Finally, Ca^{2+}-free solution containing 2 mM EGTA with 100 mM K^+ was applied for 1 min, followed by a 1 min application of Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ and the strip then brought back to Krebs solution for 20 min. These procedures were repeated in the presence of midazolam (10 and 30 μM). Midazolam was applied for 10 min in Krebs solution and was then present during the application of the various concentrations of Ca^{2+}.

Calculation of Hill coefficient

The slope of the concentration-response relationship for the effect of $[Ca^{2+}]_i$ on force is shown by the Hill coefficient (n_H) and mid-point position ($pK = (-\log K)$, where K is the dissociation constant). These parameters were obtained by fitting the data points for each curve to eqn. (1) by a non-linear least-squares method.

$$F/F_o = (C/K)^{n_H}/[1 + (C/K)^{n_H}] \qquad (1)$$

Where C represents the $[Ca^{2+}]_i$, F is the amplitude of contraction at any given $[Ca^{2+}]_i$ and F_o is the maximum response expressed as a relative force of 1.0.

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 and glucose 11.5. The concentration of K^+ was modified by the isotonic replacement of NaCl with KCl. Ca^{2+}- free Krebs solution was made by substituting an equimolar concentration of $MgCl_2$ for $CaCl_2$ and adding 2 mM EGTA. All the solutions used in the present experiments contained guanethidine (5 μM) and propranolol (3 μM) to prevent NA-outflow from sympathetic nerves and β-adrenoceptor stimulation by exogenously applied NA, respectively. The solutions were bubbled with 95% O_2 and 5% CO_2 and their pH was maintained at 7.3–7.4.

The calibration solution for Ca^{2+} measurement contained 11 mM EGTA, 110 mM KCl, 1 mM $MgCl_2$, 20 μM Fura 2 and 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (pH 7.1) with or without 11 mM $CaCl_2$.

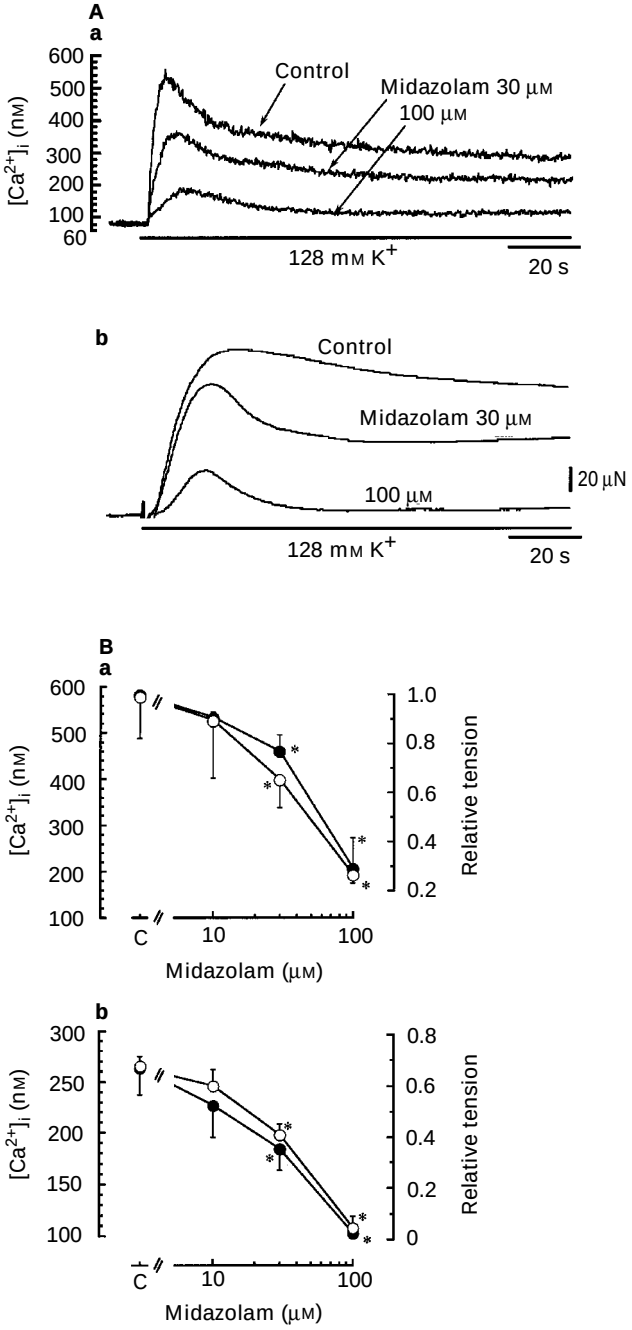

Figure 1 Effect of midazolam on increases in $[Ca^{2+}]_i$ and force induced by 128 mM K^+ in smooth muscle strips from the rabbit mesenteric artery. (A) Actual traces of simultaneous measurements of $[Ca^{2+}]_i$ (Aa) and force (Ab) obtained from a single smooth muscle strip. High K^+ (128 mM) was applied for 2 min (indicated by horizontal bars) at 20 min intervals in the presence or absence of midazolam. Midazolam was present for 10 min before and throughout the application of 128 mM K^+. (B) Concentration-dependent effects of midazolam on high K^+-induced (a) phasic and (b) tonic responses. The tonic responses were measured 2 min after the start of the application of high K^+. (○) $[Ca^{2+}]_i$; (●) relative tension, the maximum amplitude of contraction induced by 128 mM K^+ in the absence of midazolam was normalized as a relative force of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P < 0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

Drugs

Drugs used were Fura 2, Fura 2-AM, EGTA and HEPES (Dojin, Japan), caffeine (Wako, Japan), NA, nicardipine (Sigma, U.S.A.), ryanodine (Agri-system, U.S.A.), guanethidine (Tokyo Kasei, Japan), acetylcholine hydrochloride (Daiichi Pharmaceutical Co. Ltd. Japan) and propranolol

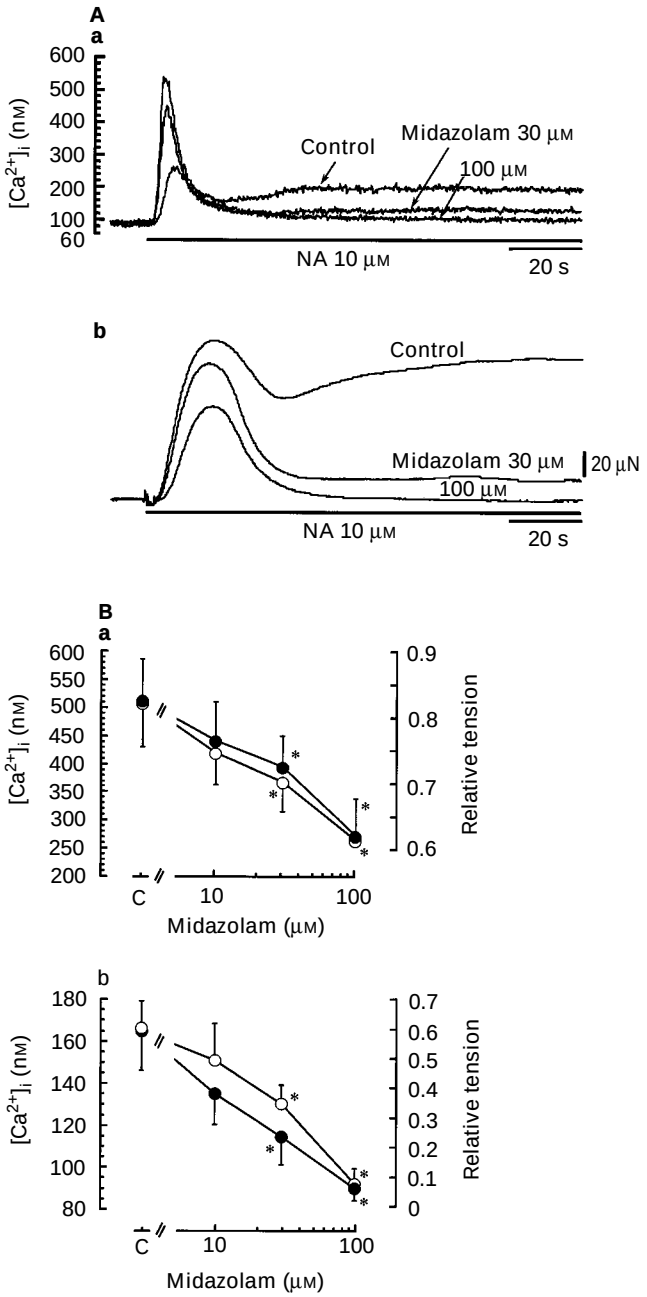

Figure 2 Effect of midazolam on increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA. (A) Actual traces of simultaneous measurements of $[Ca^{2+}]_i$ (Aa) and force (Ab) obtained from a single smooth muscle strip (the same strip as that used for Figure 1A). NA (10 μM) was applied for 2 min, as indicated by the horizontal bars. The experimental protocol was similar to that described in Figure 1. (B) Concentration-dependent effects of midazolam on (a) phasic and (b) tonic responses. The tonic responses were measured 2 min after the start of the application of NA. (○) $[Ca^{2+}]_i$; (●) relative tension, the maximum amplitude of contraction induced by 128 mM K^+ in the absence of midazolam was normalized as a relative force of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

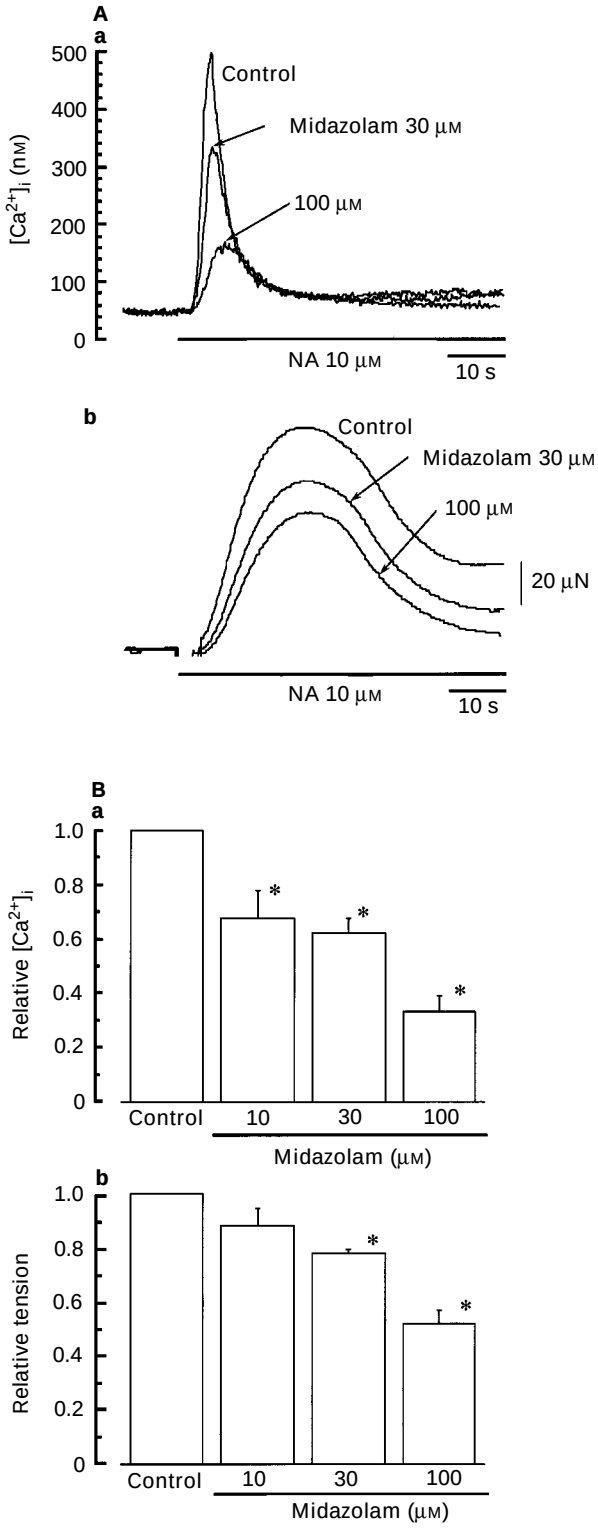

Figure 3 Effect of midazolam on increases in $[Ca^{2+}]_i$ and force induced by 10 μM NA in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. (A) Actual traces obtained from a single smooth muscle strip; $[Ca^{2+}]_i$ (Aa) and force (Ab). NA (10 μM) was applied for 2 min, as indicated by the horizontal bars. Midazolam was present for 10 min before and throughout the application of NA. (B) Concentration-dependent effect of midazolam on the NA-induced maximum responses. The maximum amplitude of each $[Ca^{2+}]_i$ and force response induced by NA in the absence of midazolam was normalized as a relative value of 1.0 for each strip. Mean of data from 4 strips, with s.e. shown by vertical lines. *Indicates values that are significantly different from control ($P<0.05$, one-way repeated-measures ANOVA and Scheffé's F test).

(Nacalai, Japan). Midazolam was kindly provided by Yamanouchi Pharmaceutical Co. Ltd. (Japan).

Statistics

The values recorded are expressed as mean ± s.e. A one-way repeated-measures ANOVA followed by Scheffé's F test for *post hoc* analysis, as well as paired or unpaired t tests were used for the statistical analysis. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Effect of midazolam on membrane potential in the presence and absence of 10 μM NA

In smooth muscle cells of the rabbit mesenteric artery, the resting membrane potential was -53.5 ± 1.7 mV ($n=12$). Midazolam (30 and 100 μM) did not alter the resting membrane potential (at 100 μM, -54.1 ± 2.1 mV) ($n=12$). NA (10 μM) depolarized the membrane to -44.5 ± 2.8 mV ($n=12$). Midazolam (100 μM) had no effect on the NA-induced membrane depolarization (-45.3 ± 1.8 mV) ($n=6$).

Effect of midazolam on the increase in $[Ca^{2+}]_i$ and force induced by high K^+, NA or caffeine

Figure 1 shows the effect of midazolam on the increase in $[Ca^{2+}]_i$ and force induced by 128 mM K^+. The resting $[Ca^{2+}]_i$ was 76 ± 10 nM at a resting force of 0.3 ± 0.4 μN ($n=4$). Application of 128 mM K^+ produced a rapid increase in $[Ca^{2+}]_i$ which reached a peak within 4–5 s (phasic phase) and then decayed to a steady level (tonic phase). The levels reached during these phases were 580 ± 92 nM and 263 ± 12 nM, respectively ($n=4$). Following the increase in $[Ca^{2+}]_i$, force developed with a phasic phase (to 124.6 ± 27.2 μN above the resting level) and a subsequent tonic phase (to 90.0 ± 23.3 μN) (Figure 1A). Midazolam (10–100 μM) did not change the resting $[Ca^{2+}]_i$ but concentration-dependently attenuated the high K^+-induced phasic and tonic increases in both $[Ca^{2+}]_i$ and force. For example, in the presence of 100 μM midazolam, the levels reached during the high K^+-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 195 ± 19 nM and 109 ± 11 nM, respectively, and the levels reached in the high K^+-induced phasic and tonic increases in force were 31.8 ± 7.0 μN and 2.7 ± 1.7 μN, respectively ($n=4$). These values were significantly different from their respective controls ($P<0.05$).

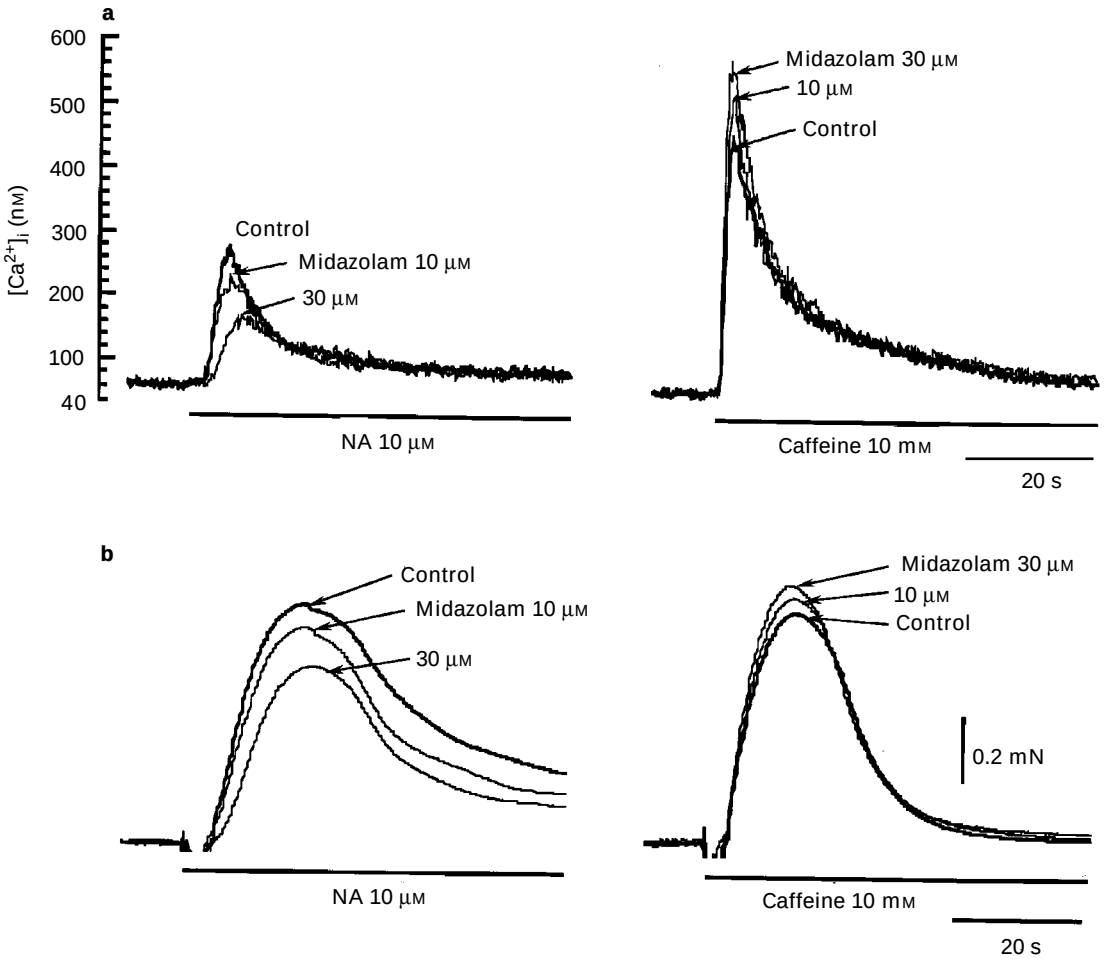

Figure 4 Concentration-dependent effect of midazolam on the increases in $[Ca^{2+}]_i$ and force induced by successively applied 10 μM NA and 10 mM caffeine in Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+. Simultaneous measurements of $[Ca^{2+}]_i$ (a) and force (b) were obtained from a single smooth muscle strip. After 2 min application of Ca^{2+}-free solution containing 2 mM EGTA, NA was first applied for 2 min (left-hand panels), followed by a 5 min washout of NA. Caffeine (right-hand panels) was then applied in Ca^{2+}-free solution and the strip then brought back to Krebs solution for 20 min. Midazolam (10 and 30 μM) was applied for 10 min before, and was present during, the application of NA. It was then washed out (for 5 min) and was not present during the subsequent application of caffeine. Thus, the effect of caffeine was tested (i) before any application of midazolam (control) and (ii) following the washout of the dose of midazolam used to test its effect on the NA-contraction.

NA (10 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force (Figure 2A). The levels reached during the NA-induced phasic increases in $[Ca^{2+}]_i$ and force were 510 ± 75 nM and 92.6 ± 12.7 μN, respectively, and during the tonic increases in $[Ca^{2+}]_i$ and force the levels reached were 165 ± 14 nM and 64.1 ± 16.1 μN, respectively ($n=4$). When midazolam (10–100 μM) was applied for 10 min before and throughout the application of 10 μM NA, this agent concentration-dependently attenuated the NA-induced phasic and tonic increases in both $[Ca^{2+}]_i$ and force. For example, in the presence of 100 μM midazolam, the levels reached during the NA-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 163 ± 5 nM and 90 ± 7 nM, respectively, and during the NA-induced phasic and tonic increases in force the levels reached were 67.2 ± 9.6 μN and 4.2 ± 2.6 μN, respectively ($n=4$). These values were significantly different from their respective controls ($P<0.05$).

Caffeine (10 mM) produced a transient increase in $[Ca^{2+}]_i$ (to 571 ± 93 nM) and force (to 107.3 ± 35.7 μN) ($n=4$). Midazolam, at low concentrations (10 and 30 μM), did not significantly modify the caffeine-induced responses, but at a higher concentration (100 μM) this agent did attenuate the caffeine-induced increases in $[Ca^{2+}]_i$ (to 487 ± 70 nM) and force (to 81.6 ± 29.2 μN) ($n=4$, $P<0.05$).

Effect of nicardipine on the increases in $[Ca^{2+}]_i$ and force induced by high K^+ or NA

Nicardipine (0.3 μM), which did not modify the resting $[Ca^{2+}]_i$ almost completely blocked the 128 mM K^+-induced increases in $[Ca^{2+}]_i$ and force. In the presence of nicardipine, the levels reached during the high K^+-induced phasic and tonic increases in $[Ca^{2+}]_i$ were 94 ± 10 nM and 79 ± 5 nM ($n=4$), respectively, and during the high K^+-induced phasic and tonic increases in force the levels were 9.1 ± 7.1 μN and 0.5 ± 0.2 μN, respectively. The corresponding control values for the phasic and tonic responses, respectively, were: for $[Ca^{2+}]_i$, 494 ± 67 nM and 192 ± 13 nM: and for force, 200.1 ± 42.6 μN and 134.1 ± 51.7 μN ($P<0.05$ in each case).

Nicardipine (0.3 μM) attenuated the tonic, but not the phasic, increases in both $[Ca^{2+}]_i$ and force induced by 10 μM NA. In control, the levels reached during the NA-induced tonic increases in $[Ca^{2+}]_i$ and force were 137 ± 10 nM and 67.9 ± 6.5 μN, respectively. In the presence of 0.3 μM nicardipine, the corresponding values were 105 ± 6 nM and 32.7 ± 8.7 μN, respectively ($n=4$, $P<0.05$). The NA-induced phasic increases in $[Ca^{2+}]_i$ and force, in the presence of nicardipine, reached 392 ± 48 nM and 122.5 ± 8.8 μN, respectively. These values were not significantly different from the corresponding controls (which were 471 ± 69 nM and 134.5 ± 12.5 μN; $P>0.05$).

Effect of midazolam on the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution

The effect of midazolam on NA-induced Ca^{2+} release was examined by observing the increase in $[Ca^{2+}]_i$ induced by 10 μM NA in Ca^{2+}-free solution containing 2 mM EGTA. The changeover of the Krebs solution to the Ca^{2+}-free solution was rapid and no increase in $[Ca^{2+}]_i$ was then induced by 128 mM K^+ after a 15 s application. Following the application of Ca^{2+}-free solution, the resting $[Ca^{2+}]_i$ rapidly decreased to 67 ± 14 nM within 1 min and then remained at this new steady level ($n=4$). Under these conditions, 10 μM NA transiently increased $[Ca^{2+}]_i$ (to 305 ± 84 nM) and force (to 149.6 ± 54.6 μN). In Ca^{2+}-free solution, midazolam (10–100 μM) attenuated the NA-evoked increases in $[Ca^{2+}]_i$ and force, in a concentration-dependent manner (Figure 3). In the presence of 100 μM midazolam, the NA-induced increases in $[Ca^{2+}]_i$ and force reached 139 ± 16 nM and 73.2 ± 22.9 μN, respectively ($n=4$); these values were significantly different from their corresponding controls ($P<0.05$). In contrast, nicardipine (0.3 μM) had no effect on the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution (data not shown).

When 10 μM NA and 10 mM caffeine were applied successively in Ca^{2+}-free solution containing 2 mM EGTA, a transient increase in $[Ca^{2+}]_i$ and force could still be induced by an application of caffeine even after an increase in $[Ca^{2+}]_i$ had been elicited by 10 μM NA (see also Itoh *et al*., 1983; 1992a). In Ca^{2+}-free solution, midazolam (10 and 30 μM) concentration-dependently attenuated the NA-induced increase in $[Ca^{2+}]_i$ and, conversely, increased the response induced by subsequently applied caffeine (Figure 4). In the presence of 30 μM midazolam, the increase in $[Ca^{2+}]_i$ induced by NA was 0.46 ± 0.07 times control ($n=4$, $P<0.05$) and that induced by subsequently applied caffeine was 1.10 ± 0.06 times control ($n=4$, $P<0.05$).

Effect of midazolam on the NA-induced increases in $[Ca^{2+}]_i$ and force in the presence of nicardipine in ryanodine-treated strips

Following an application of ryanodine (see Methods for the protocol), the resting $[Ca^{2+}]_i$ was slightly increased (from

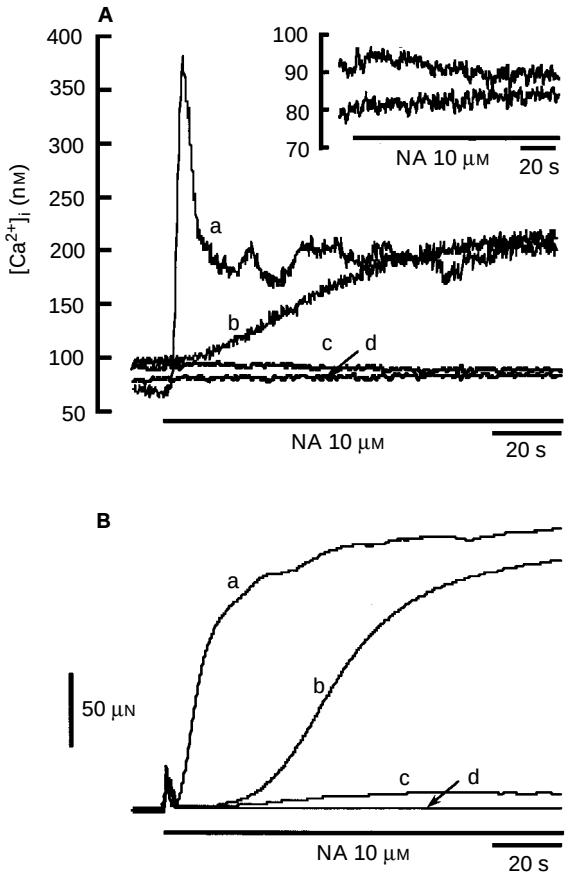

Figure 5 Effects of nicardipine and of midazolam plus nicardipine on increases in (A) $[Ca^{2+}]_i$ and (B) force induced by 10 μM NA in ryanodine-treated strips. (a) NA-induced responses before application of ryanodine. NA was applied for 3 min at 30 min intervals in Krebs solution. Between traces (a) and (b), ryanodine (50 μM) plus 10 mM caffeine was applied for 5 min in Krebs solution followed by a 10 min application of Krebs solution containing 10 μM ryanodine alone; NA was then applied again in the presence of ryanodine (b). (c) NA was applied in the presence of 0.3 μM nicardipine with ryanodine. (d) NA-response in the presence of midazolam together with nicardipine and ryanodine. Inset shows the whole of traces (c) and (d) on an expanded vertical scale (range 70–100 nM $[Ca^{2+}]_i$) but with a compressed time scale. The results illustrated were obtained from a single smooth muscle strip and were reproducible in another 3 strips.

71±7 nM to 103±8 nM, $n=6$, $P<0.05$) and subsequently applied to 10 μM NA failed to induce a phasic increase in $[Ca^{2+}]_i$ or in force ('b' in Figure 5). In fact, in ryanodine-treated strips, the increases in $[Ca^{2+}]_i$ and force induced by NA occurred slowly and the time to peak was thus greatly increased. Under these conditions, nicardipine (0.3 μM) greatly inhibited the NA-induced increase in $[Ca^{2+}]_i$ (control, 135±18 nM; nicardipine, 91±7 nM, $n=6$, $P<0.05$) with no significant change in the resting $[Ca^{2+}]_i$ ('c' in Figure. 5). In the presence of nicardipine with ryanodine, midazolam (100 μM) lowered the resting $[Ca^{2+}]_i$ (to 83±7 nM, $n=6$, $P<0.05$), though this level was still above the resting $[Ca^{2+}]_i$ and almost completely inhibited the remaining component of the increase in $[Ca^{2+}]_i$ induced by 10 μM NA ('d' in Figure 5) (81±7 nM; $n=6$, $P<0.05$).

Effect of midazolam on the $[Ca^{2+}]_i$-force relationship in the presence and absence of NA in ryanodine-treated strips

The $[Ca^{2+}]_i$-force relationship was obtained by applying various concentrations of Ca^{2+} ($[Ca^{2+}]_o$) in Ca^{2+}-free solution containing 100 mM K^+ with or without 10 μM NA in ryanodine-treated strips (see Methods for the protocol). After the application of Ca^{2+}-free solution with 5.9 mM K^+ for 1 min, the resting $[Ca^{2+}]_i$ decreased to 67 ±14 nM ($n=4$). Subsequent application of Ca^{2+}-free solution with 100 mM K^+ increased neither $[Ca^{2+}]_i$ nor force. The value of $[Ca^{2+}]_o$ required to obtain the half-maximum $[Ca^{2+}]_i$ (ED_{50}) was 425±56 μM ($n=4$) in the absence, and 502±90 μM ($n=4$) in the presence of 10 μM NA ($P>0.05$). The corresponding values of $[Ca^{2+}]_0$ for half-maximum force (ED_{50}) were 583±103 μM and 522±110 μM, respectively ($P<0.05$). The maximum values for $[Ca^{2+}]_i$ obtained on application of 2.6 mM Ca^{2+} were 270±14 nM and 269±28 nM in the absence and presence of 10 μM NA, respectively ($P>0.1$), and the corresponding values for maximum force were 140.6±23.3 μN and 205.5±22.3 μN, respectively ($P<0.05$). Under these conditions, midazolam (30 μM) significantly attenuated ($P<0.05$) the increases in $[Ca^{2+}]_i$ and force induced by any given $[Ca^{2+}]_0$ (0.16–2.6 mM) whether the experiment was conducted in the presence or absence of NA (Figure 6).

Figure 7 shows the effect of midazolam on the $[Ca^{2+}]_i$-force relationship in the presence and absence of 10 μM NA. NA shifted the $[Ca^{2+}]_i$-force relationship to the left and increased the maximum amplitude of contraction induced by 2.6 mM Ca^{2+}. The value of $[Ca^{2+}]_i$ required for half-maximum force was 211±15 nM in the absence of 10 μM NA and 166±10 nM in its presence ($P<0.05$). Midazolam (10 and 30 μM) had no effect on the $[Ca^{2+}]_i$-force relationship either in the presence or absence of NA (Figure 7).

Discussion

In the present experiments, in endothelium-denuded strips from a small resistance artery of the rabbit mesentery, midazolam (30 and 100 μM) did not modify either the resting membrane potential or the membrane depolarization induced by 10 μM NA. Midazolam (30 and 100 μM) had no effect on

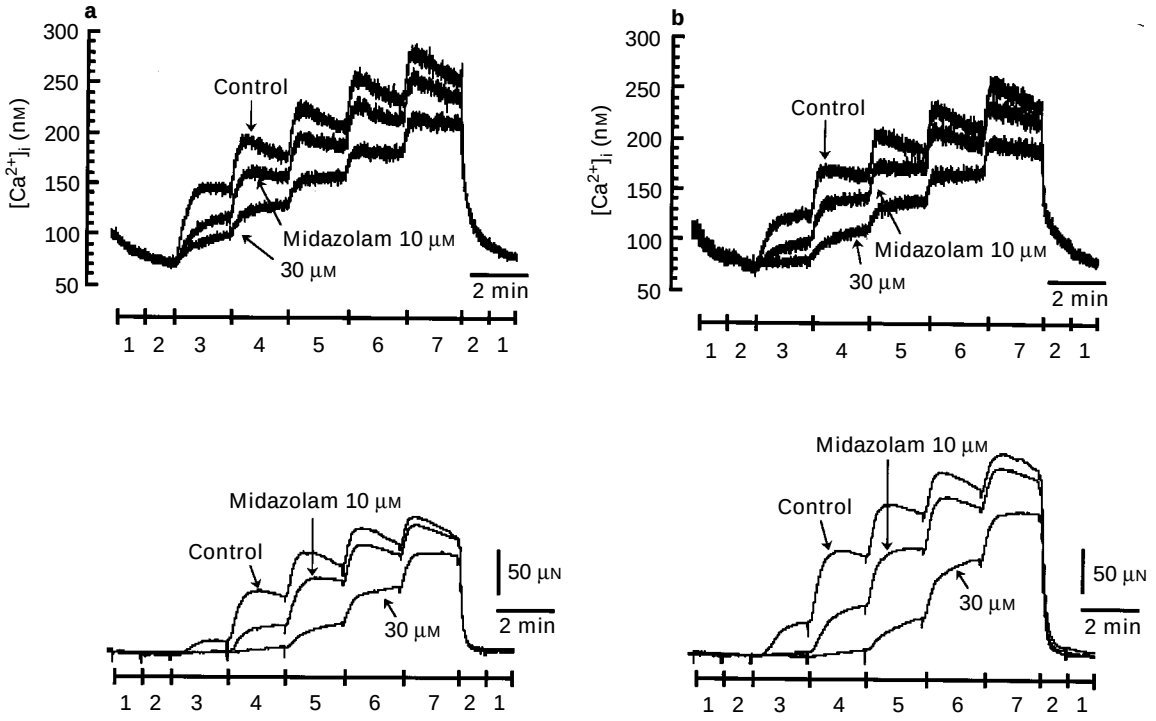

Figure 6 Effect of midazolam on increases in $[Ca^{2+}]_i$ and force induced by various concentrations of Ca^{2+} in Ca^{2+}-free solution containing 100 mM K^+ without (a) and with (b) 10 μM NA in ryanodine-treated smooth muscle strips. Ryanodine-treatment was administered as described in Figure 5. (a) Actual tracings of $[Ca^{2+}]_i$ (upper records) and force (lower records) in the absence of NA. Ca^{2+}-free solution containing 2 mM EGTA with 5.9 mM K^+ was applied for 1 min (shown as application 1) followed by a 1 min application of Ca^{2+}-free solution containing 2 mM EGTA with 100 mM K^+ (application 2). At this point, various concentrations of Ca^{2+} (0.16–2.6 mM) were cumulatively applied (each for 2 min) in an ascending order together with 100 mM K^+. These solutions contained the following: 0.16 mM Ca^{2+} (application 3), 0.33 mM Ca^{2+} (application 4), 0.65 mM Ca^{2+} (application 5), 1.3 mM Ca^{2+} (application 6) and 2.6 mM Ca^{2+} (application 7). Finally, Ca^{2+}-free solution containing 2 mM EGTA was applied to cause relaxation and the strip was then brought back to Krebs solution for 20 min. These procedures were repeated in the presence of midazolam (10 and 30 μM). Midazolam was present for 10 min in Krebs solution before, and also during the application of the various concentrations of Ca^{2+}. (b) Actual tracings of $[Ca^{2+}]_i$ (upper records) and force (lower records) in an experiment like that shown (a), but in the presence of 10 μM NA. The results were obtained from the same strip as that used for (a).

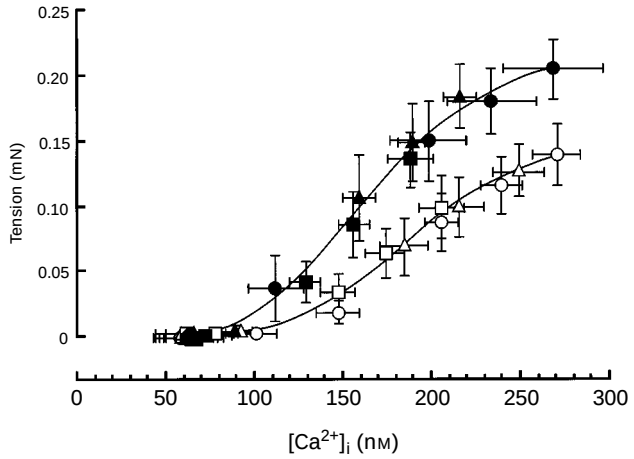

Figure 7 Effect of midazolam on $[Ca^{2+}]_i$-force relationship in the presence (solid symbols) and absence (open symbols) of 10 μM NA in high K^+, ryanodine-treated smooth muscle strips. The $[Ca^{2+}]_i$-force relationships in the presence and absence of NA were obtained as described in Figure 6. The curves were obtained by fitting the data points to eqn. (1) by a non-linear least-squares method (see Methods). Each symbol represents the mean of data from 4 strips, with s.e. shown by vertical and horizontal bars. (○, ●) Control; (△, ▲) 10 μM and (□, ■) 30 μM midazolam.

the resting $[Ca^{2+}]_i$ but greatly attenuated the increases in $[Ca^{2+}]_i$ and force induced by NA or high K^+, in a concentration-dependent manner. NA shifted the $[Ca^{2+}]_i$-force relationship to the left and increased the maximum force induced by 2.6 mM $[Ca^{2+}]_0$ in a solution containing 100 mM K^+ in ryanodine-treated strips, indicating that NA enhances the myofilament Ca^{2+}-sensitivity in the smooth muscle of the rabbit mesenteric artery, as suggested previously (Itoh *et al*., 1992a,b; 1994). Midazolam had no effect on the $[Ca^{2+}]_i$-force relationship obtained in high K^+ solution whether the tests were carried out in the presence or absence of NA in ryanodine-treated strips, suggesting that midazolam inhibits the Ca^{2+} mobilization induced by NA or high K^+, and in this way attenuates the contractions induced by these stimulants.

It is thought that the membrane depolarization induced by high K^+ activates Ca^{2+} influx through the L-type Ca^{2+} channel and thus causes contraction (Kuriyama *et al*., 1995). In the present experiments, nicardipine (0.3 μM, a blocker of the L-type Ca^{2+} channel) inhibited the high K^+-induced increases in $[Ca^{2+}]_i$ and force. Under the present conditions, midazolam (30 and 100 μM) also greatly attenuated the high K^+-induced increases in $[Ca^{2+}]_i$ and force. Thus, in good agreement with previous findings (French *et al*., 1989; Chang *et al*., 1994), this result suggests that midazolam inhibits voltage-activated Ca^{2+} influx through the L-type Ca^{2+} channel.

In the present experiments on the smooth muscle of the rabbit mesenteric artery, NA (10 μM) produced a phasic, followed by a tonic increase in both $[Ca^{2+}]_i$ and force. NA and caffeine each produced phasic increases in $[Ca^{2+}]_i$ and force that were large and transient whether in the presence or absence of extracellular Ca^{2+}. It has previously been shown that, in this tissue, caffeine releases Ca^{2+} from stores that are closely related to the NA-sensitive ones, but that the effects are exerted via different mechanisms (Itoh *et al*., 1983). In good agreement with our previous findings (Itoh *et al*., 1992a), ryanodine abolished the phasic increases in $[Ca^{2+}]_i$ and force induced by both NA and caffeine in the presence and absence of Ca^{2+}. Thus, these results suggest that, in smooth muscle of the rabbit mesenteric artery, NA releases Ca^{2+} from the ryanodine-sensitive Ca^{2+} storage sites, although it is known to produce tonic increases in $[Ca^{2+}]_i$ and force via activation of Ca^{2+} influx (Itoh *et al*., 1983; 1992a; 1994). We previously found that in ryanodine-treated smooth muscle of the rabbit mesenteric artery, which has functionally lost the NA-sensitive Ca^{2+}-storage sites (as described above), the membrane hyperpolarization induced by K_{ATP}-channel openers almost completely inhibited the NA-induced tonic increases in $[Ca^{2+}]_i$ and force,whereas nicardipine (0.3–1 μM) only partially inhibited it (Itoh *et al*., 1994). This suggests that NA increases Ca^{2+} influx not simply by activating L-type Ca^{2+} channels in this tissue; additional mechanisms are presumably activated, too.

Effects of midazolam on NA-induced Ca^{2+} influx

In smooth muscle cells, Ca^{2+} influx can be increased by agonists through activation of both the dihydropyridine-sensitive and -insensitive, receptor-operated non-selective cation channels (Benham & Tsien, 1987; Inoue & Isenberg, 1990; Pacaud & Bolton, 1990). In the present experiments, 0.3 μM nicardipine almost completely blocked the increases in $[Ca^{2+}]_i$ and force induced by 128 mM K^+. This suggests that, at this concentration, this agent can effectively inhibit L-type Ca^{2+} channel activity in rabbit mesenteric arterial smooth muscle. Nicardipine (0.3 μM) also attenuated the NA-induced tonic increase in $[Ca^{2+}]_i$, although the magnitude of this inhibition was slightly, but significantly, smaller than that induced by 100 μM midazolam ($P<0.05$).

How might these results be best explained? The NA-induced tonic increase in $[Ca^{2+}]_i$ is thought to be provoked by an interplay between NA-induced Ca^{2+} influx and Ca^{2+} release from the storage sites (Itoh *et al*., 1983; 1992b). In ryanodine-treated smooth muscle strips from the rabbit mesenteric artery, NA failed to produce a phasic increase in $[Ca^{2+}]_i$ and only produced a mono-tonic response,which was abolished in Ca^{2+}-free solution (Itoh *et al*., 1992a,b). These results indicate that in ryanodine-treated smooth muscle, NA increases $[Ca^{2+}]_i$ only through a NA-activated Ca^{2+} influx. To further test whether or not the attenuation by midazolam of the NA-induced Ca^{2+} influx is solely due to an inhibition of L-type Ca^{2+} channels, the effect of midazolam was studied on the NA-induced increase in $[Ca^{2+}]_i$ and force seen in ryanodine-treated muscle strips. In such strips, nicardipine (0.3 μM) attenuated the NA-induced increases in $[Ca^{2+}]_i$ and force, and midazolam (100 μM) further attenuated the NA-induced increase in $[Ca^{2+}]_i$ and force that remained in the presence of nicardipine. These results suggest that midazolam inhibits the NA-induced Ca^{2+} influx through inhibition of both dihydropyridine-sensitive and -insensitive Ca^{2+} influxes in smooth muscle of the rabbit mesenteric artery.

Effects of midazolam on NA-induced Ca^{2+}-release

Midazolam (10 and 30 μM) concentration-dependently attenuated the NA-induced increases in $[Ca^{2+}]_i$ and force in Ca^{2+}-free solution. However, the same concentrations of midazolam had no effect on the caffeine-induced increases in $[Ca^{2+}]_i$ and force. These results suggest that midazolam selectively inhibits the NA-induced Ca^{2+} release from the storage sites. This conclusion is supported by the present finding that, when NA and caffeine were applied successively in Ca^{2+}-free solution, midazolam (10 and 30 μM) enhanced the caffeine-induced increase in $[Ca^{2+}]_i$ while, at the same time, attenuating the NA-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner. These results suggest that the attenuating effect of midazolam on the NA-induced increase in $[Ca^{2+}]_i$ in Ca^{2+}-free solution may be due to an inhibitory effect on the NA-induced Ca^{2+} release from the storage sites, rather than to any enhancement of Ca^{2+} extrusion through the plasma membrane.

It is known that NA binds to α-receptors and synthesizes $InsP_3$; this is thought to play an essential role in NA-induced Ca^{2+} release in smooth muscle of the rabbit mesenteric artery (Hashimoto *et al*., 1986; Itoh *et al*., 1992b). It is unlikely that midazolam acts as an α-receptor blocker since this agent had no effect on the NA-induced membrane depolarization, an action of NA which is blocked by prazosin, an α-receptor blocker. Midazolam has been shown to hyperpolarize the plasma membrane of cells in the central nervous system, in

addition to its effect on the GABA receptor complex, and the former effect occurs at doses smaller than those needed to enhance the actions of GABA (Carlen *et al*., 1983). In smooth muscle of the rabbit mesenteric artery, we previously found that the membrane hyperpolarization induced by K_{ATP}-channel openers inhibits the NA-induced $InsP_3$ production and attenuates the NA-induced release of Ca^{2+} from the storage sites (Ito *et al*., 1991; Itoh *et al*., 1992a; 1994). However, in the present study, midazolam did not modify the membrane potential either in the presence or absence of NA. Thus, membrane hyperpolarization is not the mechanism responsible for the midazolam-induced inhibition of NA-induced Ca^{2+} release. The precise details of the mechanism actually responsible in resistance arteries of the rabbit mesentery will need to be clarified in future work.

It has been shown that benzodiazepines (diazepam, flunitrazepam and midazolam) relax tracheal smooth muscle, and that this is not mediated via neural pathways or via central or peripheral benzodiazepine receptors, but is a direct action on airway smooth muscle (Koga *et al*., 1992; Yoshimura *et al*., 1995). In porcine tracheal smooth muscle, Yoshimura *et al*., (1995) found that midazolam inhibits the contractions induced by high K^+ and carbachol by decreasing $[Ca^{2+}]_i$ without changing the sensitivity of the contractile proteins to $[Ca^{2+}]_i$. These authors also found that midazolam did not alter the carbachol-induced release of stored Ca^{2+}, and thus suggested that midazolam inhibits carbachol-induced contraction through inhibition of Ca^{2+} influx in porcine tracheal smooth muscle. This finding differs from our results in NA-stimulated smooth muscle of the rabbit mesenteric artery. At present, we do not know the reason for this discrepancy, but the different actions of midazolam on agonist-induced Ca^{2+} release might be due to species or regional differences or to the different agonists used (i.e. carbachol and NA). This needs to be clarified in future work.

It is concluded that, in the smooth muscle of rabbit mesenteric resistance arteries, midazolam inhibits the increases in $[Ca^{2+}]_i$ and force induced by NA or high K^+ without producing a change in the $[Ca^{2+}]_i$-force relationship. Midazolam attenuates the high K^+-induced increases in $[Ca^{2+}]_i$ and force via inhibition of Ca^{2+} influx through the L-type Ca^{2+} channel. In contrast, it attenuates the NA-induced phasic and tonic increases in $[Ca^{2+}]_i$ and force through inhibition not only of NA-induced Ca^{2+} release, but also of NA-induced Ca^{2+} influx. The latter effect is induced via inhibition of both nicardipine-sensitive and -insensitive pathways.

We thank Dr R.J. Timms for a critical reading of the English. This work was partly supported by a Grant-In-Aid from the Ministry of Education of Japan. Midazolam was a gift from Yamanouchi Pharmaceutical Co. Ltd. (Japan).

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(Received February 3, 1997
Revised March 25, 1997
Accepted April 7, 1997)