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Effects of Midazolam on Intracellular Ca^{2+} and Tension in Airway Smooth Muscles

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Background: **It has been demonstrated that a group of minor tranquilizers, benzodiazepines, are able to relax airway smooth muscles. To determine the underlying mechanisms of this phenomenon, the effects of midazolam on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and tension in airway smooth muscles were investigated.**

Methods: **Using front-surface fluorometry and fura-2-loaded porcine tracheal smooth muscle strips, both $[Ca^{2+}]_i$ and isometric tension developments were simultaneously recorded.**

Results: **When the tracheal strips were exposed to a high external K^+-solution (40 mM) or 10^{-7} M carbachol containing 1.25 mM Ca^{2+}, both $[Ca^{2+}]_i$ and tension increased rapidly until they reached a plateau (the steady state). During steady-state contraction induced by K^+-depolarization or carbachol, the cumulative application of midazolam ($10^{-7} \sim 10^{-4}$ M) caused decreases in both $[Ca^{2+}]_i$ and tension, in a concentration-dependent manner. During 40 mM K^+-induced depolarization, the stepwise increases in the extracellular Ca^{2+} concentration induced the stepwise increases in $[Ca^{2+}]_i$ and tension. Midazolam (3×10^{-5} M) inhibited these increases in $[Ca^{2+}]_i$ and tension, but had no effect on the $[Ca^{2+}]_i$-tension relationship. In the presence of 3×10^{-3} M $NiCl_2$ (a nonselective cation channel blocker), midazolam (3×10^{-5} M) did not cause any additional reduction of $[Ca^{2+}]_i$ or tension during the contraction induced by carbachol (10^{-7} M). In the absence of extracellular Ca^{2+}, midazolam (3×10^{-5} M) had no effect on the transient increases in either $[Ca^{2+}]_i$ or the tension induced by carbachol (10^{-7} M) or caffeine (20 mM). Pretreatment with both 10^{-5} M flumazenil (a specific central antagonist of benzodiazepines) and 10^{-5} M PK11195 (a specific peripheral antagonist of benzodiazepines) did not influence the effect of 10^{-5} M midazolam on $[Ca^{2+}]_i$ or tension during the contractions induced by carbachol.**

Conclusions: **Midazolam directly relaxes airway smooth muscles by decreasing $[Ca^{2+}]_i$; this can be attributed to the inhibition of the influx of extracellular Ca^{2+}. Midazolam has no effect on the release of stored Ca^{2+}. In addition, midazolam has no effect on Ca^{2+} sensitivity of the contractile apparatus. Finally, benzodiazepine antagonists, flumazenil and PK11195, have no effect on this mechanism of direct action of midazolam on airway smooth muscles. (Key words: Airway: trachea. Antagonists, benzodiazepines: flumazenil. Hypnotics: midazolam. Ions, calcium: intracellular Ca^{2+} transient. Muscle: smooth.)**

MIDAZOLAM has been widely used as a sedative and an induction agent of general anesthesia. In addition to the hypnotic action, it has also been reported that midazolam causes vasodilation[1–4] and relaxes airway smooth muscle.[5–7] Midazolam's effect on the airway smooth muscle is favorable for patients who demonstrate airway hypersensitivity. However, there has been no report about the mechanism of the relaxant effects of midazolam on airway smooth muscle.

Although it is generally accepted that smooth muscle tone is primarily regulated by cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), the development and maintenance of tension does not simply depend on $[Ca^{2+}]_i$.[8] Recently, it has been shown that in α-toxin- or β-escin-permeabilized smooth muscles, some agonists induced an enhanced sensitivity of myofilament to Ca^{2+}, through a

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G-protein-mediated pathway.[9–10] In addition, it has also been shown that contractions can occur without any change in $[Ca^{2+}]_i$ in intact smooth muscle strips.[11] Thus, to determine the mechanism underlying the changes in smooth muscle tone, it is important to determine the change in $[Ca^{2+}]_i$ in association with the change in tension. In this study, we investigated the mechanism of midazolam-induced relaxation of airway smooth muscle by simultaneously measuring the tension and $[Ca^{2+}]_i$, using front-surface fluorometry[12,13] and the Ca^{2+} indicator dye fura-2. The questions asked in this study were whether or not: (1) midazolam reduces $[Ca^{2+}]_i$; (2) midazolam changes the sensitivity of the contractile apparatus to $[Ca^{2+}]_i$; and (3) flumazenil (specific central benzodiazepine antagonist)[14–17] or PK11195 (specific peripheral benzodiazepine antagonist)[1,18,19] influence the effect of midazolam on airway smooth muscle.

Materials and Methods

Tissue Preparation

Tracheas were dissected from adult pigs at a local slaughterhouse using a protocol approved by the Animal Research Committee of Research Institute of Angiocardiology, Faculty of Medicine, Kyushu University. The tracheas were placed in ice-cold physiologic salt solution (PSS) and brought to our laboratory. The lower end of the trachea, just above the first bronchus branching, three tracheal rings in length, was used for the experiments. The posterior portion of the trachea was excised longitudinally, and all cartilage was detached. The mucosa and adventitial tissue were removed under microscopic observation. The muscle sheets were cut transversely into rectangular strips approximately 3 mm long and 1 mm wide.[20] All tissue preparations in the laboratory were performed in aerated PSS.

Fura-2 Loading

Tracheal strips were loaded with the Ca^{2+} indicator dye, fura-2, in the form of acetoxymethyl ester (fura-2/AM). The strips were incubated in 1 ml aerated (95% O_2: 5% CO_2) Dulbecco-modified Eagle's medium containing 50 μM Fura-2/AM and 5% fetal bovine serum for 3 hr at 37°C. After loading with fura-2, the strips were washed with normal PSS to remove dye in the extracellular space, and then were further incubated in normal PSS for at least 1 hr to facilitate the deesterification of intracellular fura-2/AM and to equilibrate the strips before taking the measurements.[20]

Measurement of Tension Development

Each strip was mounted vertically in a 6-ml quartz organ bath, which was maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. The lower end of the strip was fixed by a small clip and the upper end of the strip was attached by a small clip and thread to a force transducer (TB-612T, Nihon Koden, Japan) to record the isometric tension. During the 1-hr fura-2 equilibration period, the strips were stimulated with 40 mM K^+ PSS at 5–10-min intervals, and muscle length was increased stepwise after each stimulation until the developed tension reached a maximum. When exposed to 40 mM K^+ PSS, most strips produced stable tension within 1 min. The strips that showed an instability in tension as induced by 40 mM K^+ PSS, were excluded from the study. The responsiveness of each strip to 40 mM K^+ PSS was then recorded before starting the experimental protocol, because almost the maximum, reproducible responses of tension to high K^+ depolarization were obtained at this concentration of K^+. The developed tension was expressed as a percentage, assuming the values in normal (5.9 mM K^+) PSS and steady state of 40 mM K^+ PSS to be 0% and 100%, respectively.

Measurement of Fura-2 Fluorescence

Changes in the fluorescence intensity of the fura-2–Ca^{2+} complex were monitored using a front-surface fura-2 fluorometer (model CAM-OF Co.), specifically designed in collaboration with Japan Spectroscopic (Tokyo, Japan). The details of our front-surface fluorometry system have been described elsewhere.[11–13,] In brief, two wavelengths of excitation light (340 and 380 nm) were obtained spectroscopically from a Xenon light source. The strips were illuminated by guiding the two alternating (400-Hz) wavelengths of excitation light through quartz optic fibers. The surface fluorescence of the strip was collected by glass optic fibers and introduced through a 500-nm band-pass filter into a photomultiplier. Thus, we measured the fura-2 fluorescence intensity of 500-nm emission light, which was induced by alternating two wavelengths of excitation light (340 and 380 nm).

The ratio of the fluorescence intensities (fluorescence ratio) at 340 nm excitation to that at 380 nm excitation was monitored to estimate changes in $[Ca^{2+}]_i$ and expressed as a percentage, assuming the values in normal PSS (5.9 mM K^+) and steady state of 40 mM K^+ PSS to

be 0% and 100%, respectively. The absolute values of $[Ca^{2+}]_i$ for 0% and 100% levels of the fluorescence ratio were determined separately using the following protocol and the equation of Grynkiewicz *et al.*[21]: After recording 0% and 100% levels of the fluorescence ratio, the minimum and the maximum fluorescence ratios were determined by the addition of 25 μM ionomycin to Ca^{2+}-free PSS containing 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA), followed by replacement with normal PSS (1.25 mM Ca^{2+}), respectively. The absolute values of $[Ca^{2+}]_i$ in normal PSS (0%) and the steady state of 40 mM K^+ PSS (100%) were calculated and they were 90 ± 14 and 499 ± 54 nM (n = 8), respectively. Thus, levels of $[Ca^{2+}]_i$ for each experiment were expressed as percent levels of the fluorescence ratio, and the absolute values of $[Ca^{2+}]_i$ for these percent levels were shown in the right ordinate of figures and parenthetically in the text as references.

Experimental Protocols

To examine the effect of midazolam on $[Ca^{2+}]_i$ and tension during contractions, midazolam ($10^{-7} \sim 10^{-4}$ M) was cumulatively applied on the steady state of elevations of $[Ca^{2+}]_i$ and tension induced by 40 mM K^+ PSS or 10^{-7} M carbachol.

To examine the effect of antagonists against benzodiazepines on the effect of midazolam, strips were treated with flumazenil or PK11195. Flumazenil (10^{-5} M) or PK11195 (10^{-5} M) were applied for 5 min before and during the application of 10^{-7} M carbachol. Midazolam (10^{-5} M) was then applied at the steady state of the contraction induced by carbachol.

To examine the effect of midazolam on Ca^{2+} sensitivity of the contractile apparatus, we determined the $[Ca^{2+}]_i$-tension relationships in the contractions induced by the stepwise increases in the extracellular Ca^{2+} concentration during 40 mM K^+-induced depolarization, in the absence or presence of 10^{-7} M carbachol, as follows: After 10 min incubation in Ca^{2+}-free PSS containing 2 mM EGTA, and then 5 min incubation in Ca^{2+}-free PSS without EGTA, strips were immersed in Ca^{2+}-free 40 mM K^+ solution. Then, the extracellular Ca^{2+} concentration was increased by the cumulative addition of $CaCl_2$. Midazolam (3×10^{-5} M) was applied at the time of replacement with Ca^{2+}-free PSS without EGTA. To determine the effect of midazolam on the contractions in the presence of carbachol, 10^{-7} M carbachol was applied after 5 min incubation in Ca^{2+}-free PSS containing 2 mM EGTA.

To examine the effect of midazolam on the dynamic changes in $[Ca^{2+}]_i$ and tension during the contraction induced by carbachol, we observed the time courses of changes in $[Ca^{2+}]_i$ and tension induced by 10^{-7} M carbachol in strips being treated with midazolam (0, 3×10^{-5} M) for 10 min before and during the application of carbachol.

To examine the effect of midazolam on Ca^{2+} release from the intracellular store sites, two different experiments were performed, in which the Ca^{2+} entry from the extracellular space was eliminated; the first is the inhibition of Ca^{2+} influx and the second is the elimination of extracellular Ca^{2+}. First, the effect of midazolam on Ca^{2+} release from the intracellular store during the inhibition of Ca^{2+} influx was determined. To inhibit the influx of Ca^{2+} from the extracellular space, 3×10^{-3} M $NiCl_2$ (an inorganic Ca^{2+} entry blocker[22]) was added 15 min before the application of 10^{-7} M carbachol. Midazolam was applied 10 min before the application of carbachol. Second, the effect of midazolam on the Ca^{2+} release by caffeine or carbachol was determined as follows; after 10 min incubation in Ca^{2+}-free PSS containing 2 mM EGTA, 20 mM caffeine or 10^{-7} M carbachol was applied. In midazolam-treated strips, 3×10^{-5} M midazolam was applied 5 min before and throughout the application of caffeine or carbachol.

Solutions and Drugs

The normal PSS consisted of the following composition (in mM): NaCl 123, KCl 4.7, $NaHCO_3$ 15.5, KH_2PO_4 1.2, $CaCl_2$ 1.25, and D-glucose 11.5. High K^+ PSS was identical to normal PSS, except for an equimolar substitution of KCl for NaCl. The Ca^{2+}-free version of PSS was produced by the exclusion of $CaCl_2$ from the composition of normal PSS. PSS was bubbled with 95% O_2 and 5% CO_2, with a resulting pH of 7.4 at 37°C. Fura-2/AM and EGTA were purchased from Dojindo (Kumamoto, Japan). The midazolam and flumazenil were donated by Yamanouchi Pharmaceutical Co. (Tokyo, Japan). The carbachol was obtained from Sigma Chemical (St. Louis, MO), and the caffeine was obtained from Katayama Chemical (Osaka, Japan), and the PK11195 was obtained from Research Biochemicals Inc. (Natick, MA).

Data Analysis

The measured values were expressed as the mean ± SE (n = number of observations). A repeated-measures one-way analysis of variance was used to determine the concentration-dependent effects. A repeated-measures

two-way analysis of variance for was used to determine the statistical significance of the effect of midazolam pretreatment on the contractions induced by extracellularly applied Ca^{2+} during high K^+ depolarization. Analysis of covariance was used to determine the statistical significance of the shift of the $[Ca^{2+}]_i$-tension relationship. For the rest of the measurements, an unpaired Student's *t* test was used. *P* values of less than 0.05 were considered to be significant. The IC_{50} values (the midazolam concentration that decreases $[Ca^{2+}]_i$ and tension to 50% of the maximal response) were calculated, using the four-parameter logistic equation reported by De Lean *et al.*[23]

Results

The Effect of Midazolam on $[Ca^{2+}]_i$ and Tension during the Contraction Induced by Depolarization with High K^+ Solution

As shown in figure 1A, when the tracheal strip was depolarized by the exposure to 40 mM K^+ PSS in the presence of 1.25 mM Ca^{2+}, $[Ca^{2+}]_i$ and tension abruptly increased until reaching a peak, and then was sustained either at this level or at slightly decreased levels until reaching a plateau (or a steady state). When midazolam was cumulatively applied ($10^{-7} \sim 10^{-4}$ M) during the steady state of the contraction, a concentration-dependent reduction in $[Ca^{2+}]_i$ and tension occurred (Figs. 1A and 1B). The application of 10^{-4} M midazolam reduced $[Ca^{2+}]_i$ and tension to 18.1 ± 5.2% (122 nM) and 6.5 ± 2.1%, respectively (n = 6). The IC_{50} values for $[Ca^{2+}]_i$ and tension were approximately 4.1×10^{-5} M and 2.1×10^{-5} M, respectively. When midazolam ($10^{-7} \sim 10^{-4}$ M) was cumulatively applied during the resting state in normal PSS, no significant change in $[Ca^{2+}]_i$ or tension was detected (data not shown). After washing out midazolam with normal PSS for 10 min, 40 mM K^+ caused the same measure of response in $[Ca^{2+}]_i$ and tension, thereby indicating that the effect of midazolam ($\leq 10^{-4}$ M) examined in this study was reversible.

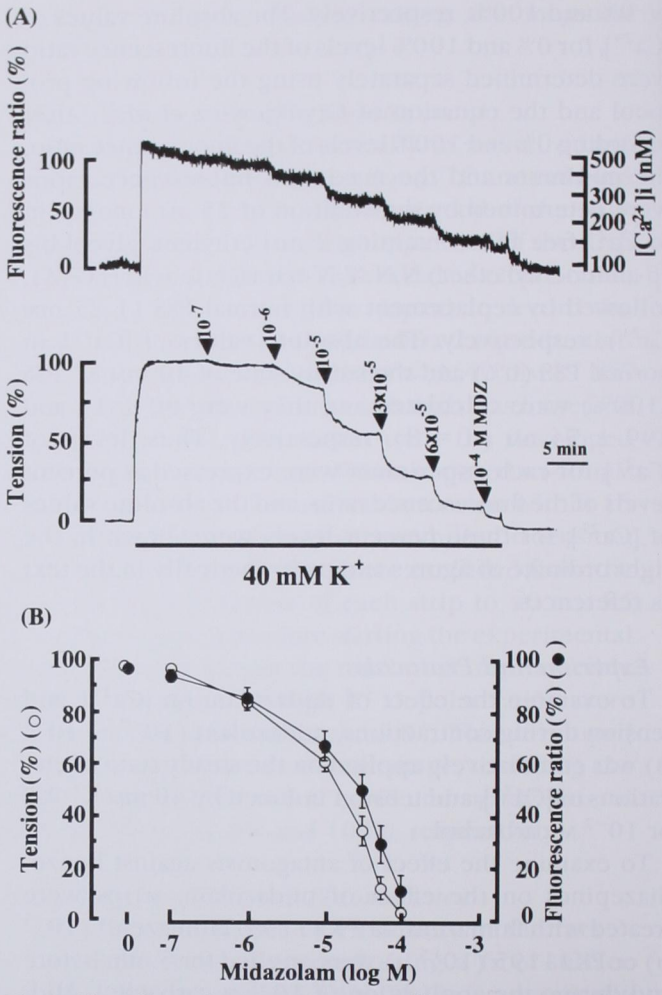

Fig. 1. The effect of midazolam on $[Ca^{2+}]_i$ and tension during the contraction induced by depolarization with a high K^+ solution. (*A*) Representative recording showing the effect of cumulatively applied midazolam ($10^{-7} \sim 10^{-4}$ M) on $[Ca^{2+}]_i$ and tension during the contractions induced by 40 mM K^+ depolarization. At the point indicated by an arrowhead, midazolam was applied to make the final concentration shown in the figure. (*B*) The concentration-dependent effect of midazolam on elevated $[Ca^{2+}]_i$ (●) and tension (○) induced by 40 mM K^+ depolarization. The fluorescence ratio and tension were expressed as a percentage by assuming the values at normal physiologic salt solution (5.9 mM K^+) and the steady state during the 40 mM K^+-depolarization to be 0 and 100% respectively. The plots represent the means of six preparations, with the standard error shown by vertical bars.

The Effect of Midazolam on $[Ca^{2+}]_i$ and Tension during Contractions Induced by Carbachol

Figure 2A is a representative recording showing the effect of a cumulative application of midazolam ($10^{-7} \sim 6 \times 10^{-5}$ M) on $[Ca^{2+}]_i$ and tension during the contraction induced by 10^{-7} M carbachol. After recording the 100% response levels by the depolarization with 40 mM K^+ PSS, carbachol was applied. Carbachol induced a rapid rise (the first component) in $[Ca^{2+}]_i$ and tension followed by a gradual decrease to a steady-state level (the second component) within 10 min. These steady levels of $[Ca^{2+}]_i$ [58.5 ± 9% (259.1 nM)] and tension (118 ± 12%) (n = 5) were then maintained

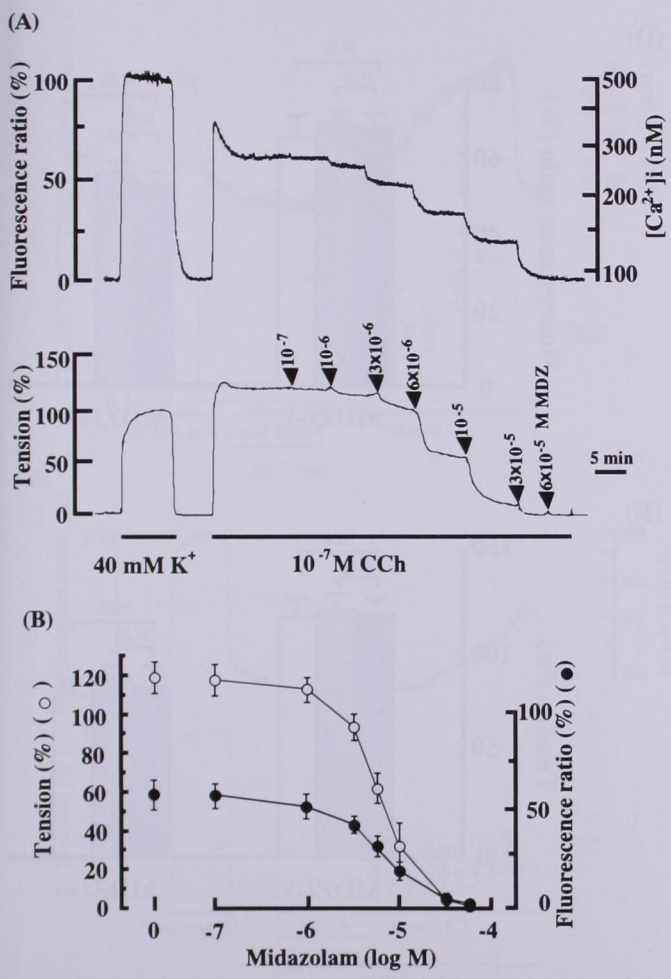

Fig. 2. The effect of midazolam on $[Ca^{2+}]_i$ and tension during the contraction induced by 10^{-7} M carbachol. (*A*) Representative recording showing the effect of cumulatively applied midazolam ($10^{-7} \sim 6 \times 10^{-5}$ M) on $[Ca^{2+}]_i$ and tension during the contractions induced by 10^{-7} M carbachol. (*B*) The concentration-dependent effect of midazolam on $[Ca^{2+}]_i$ (●) and tension (○) during the contraction induced by 10^{-7} M carbachol. The fluorescence ratio and tension were expressed as a percentage by assuming the values at normal physiologic salt solution (5.9 mM K^+) and the steady state during 40 mM K^+ depolarization to be 0 and 100%, respectively. The plots represent the means of five preparations, with the standard error shown by vertical bars.

during the 1-hr observation period. The cumulative application of midazolam, at the steady level of contraction induced by carbachol, caused a concentration-dependent reduction in $[Ca^{2+}]_i$ and tension. The application of 6×10^{-5} M midazolam reduced $[Ca^{2+}]_i$ and tension to 2.31 ± 0.9% (95.1 nM) and 4.1 ± 1.1%, respectively (n = 6). The IC_{50} values for $[Ca^{2+}]_i$ and tension were approximately 6.5×10^{-6} M and 6.3×10^{-6} M, respectively.

Effects of Flumazenil and PK11195

Figure 3A is a representative recording of the effect of application of 10^{-5} M midazolam on elevated $[Ca^{2+}]_i$ and tension induced by 10^{-7} M carbachol. $[Ca^{2+}]_i$ and tension were rapidly reduced to reach steady-state levels by midazolam. Neither the treatment with 10^{-5} M flumazenil (Fig. 3B) nor the treatment with 10^{-5} M PK11195 (Fig. 3C) for 5 min before and during the application of carbachol influenced carbachol-induced elevation of $[Ca^{2+}]_i$ and tension, nor did it effect midazolam-induced reduction of elevated $[Ca^{2+}]_i$ and tension (Figs. 3D and 3E).

Effects of Midazolam on the Increases in $[Ca^{2+}]_i$ and Tension Induced by Increases in Extracellular Ca^{2+} Concentration during High K^+-Depolarization

Figure 4A shows a representative recording of changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ during depolarization with 40 mM K^+. In response to the stepwise increment of extracellular Ca^{2+} concentration (0.0125–2.5 mM), $[Ca^{2+}]_i$ and tension increased in a concentration-dependent manner. When the extracellular Ca^{2+} was 2.5 mM, $[Ca^{2+}]_i$ and tension were 105.6 ± 7.2% (548 nM) and 85.3 ± 10.5%, respectively (n = 10). Treatment with 3×10^{-5} M midazolam for 8 min before and during the cumulative application of extracellular Ca^{2+} significantly inhibited increases in $[Ca^{2+}]_i$ and tension ($P < 0.01$ for both, by two-way analysis of variance) (Fig. 4B). In the midazolam-treated strips, when the extracellular Ca^{2+} was 5 mM, $[Ca^{2+}]_i$ and tension were 61.8 ± 8.6% (273.6 nM) and 20.8 ± 8.2% respectively (n = 6).

Effect of Midazolam on the Increases in $[Ca^{2+}]_i$ and Tension Induced by Increases in Extracellular Ca^{2+} Concentration during High K^+-Depolarization in the Presence of Carbachol

Figure 5A shows a representative recording of changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ during depolarization with 40 mM K^+ in the presence of 10^{-7} M carbachol. In response to the stepwise increment of extracellular Ca^{2+} concentration (0.0125–1.25 mM), $[Ca^{2+}]_i$ and tension increased in a concentration-dependent fashion. When the extracellular Ca^{2+} was 1.25 mM, $[Ca^{2+}]_i$ and tension were 72.4 ± 8.2% (322.8 nM) and 206.3 ± 14.2%,

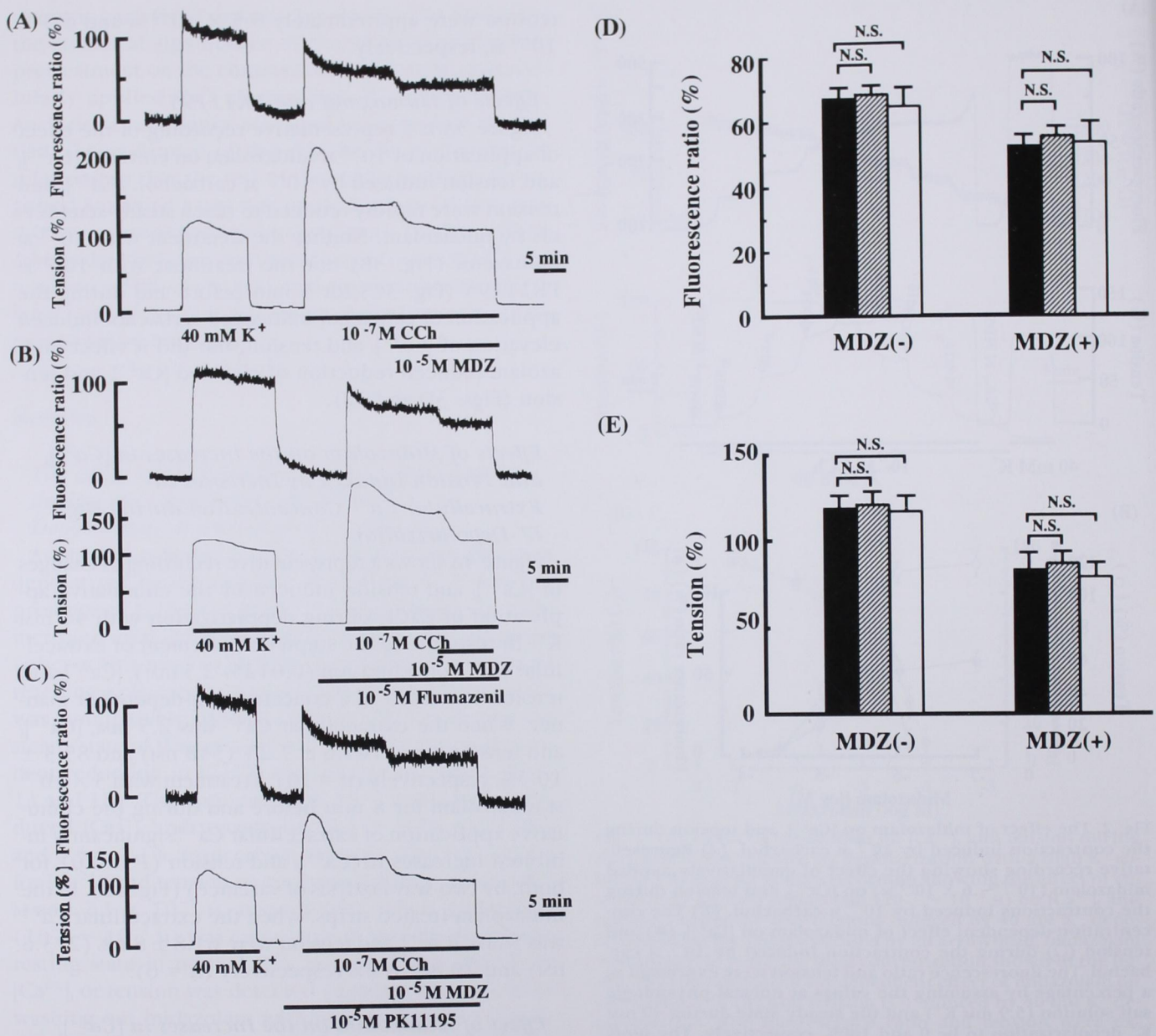

Fig. 3. Effect of flumazenil and PK11195 on the decreases in $[Ca^{2+}]_i$ and tension induced by 10^{-5} M midazolam. (*A*) Representative recording showing the effect of 10^{-5} M midazolam on $[Ca^{2+}]_i$ and tension development induced by 10^{-7} M carbachol. (*B*) Representative recording showing the effect of the treatment with 10^{-5} M flumazenil on the midazolam-induced decreases in $[Ca^{2+}]_i$ and tension during 10^{-7} M carbachol-induced contraction. (*C*) Representative recording showing the effect of the treatment with 10^{-5} M PK11195 on the midazolam-induced decreases in $[Ca^{2+}]_i$ and tension during 10^{-7} M carbachol-induced contraction. (*D*) $[Ca^{2+}]_i$ and (*E*) tension development before (*left*) and after (*right*) the application of midazolam, in the absence (closed columns) and the presence of 10^{-5} M flumazenil (hatched columns) or 10^{-5} M PK11195 (open columns). The vertical bars at the top of each column show standard error (n = 5).

respectively (n = 10). Treatment with 3×10^{-5} M midazolam for 8 min before and during the cumulative application of extracellular Ca^{2+} significantly inhibited increases in $[Ca^{2+}]_i$ and tension ($P < 0.01$ for both, by two-way analysis of variance; Fig. 5B). In the midazolam-treated strips, when the extracellular Ca^{2+} was 2.5 mM, $[Ca^{2+}]_i$ and tension were 55.5 ± 7.6% (247.8 nM) and 129.3 ± 14.3%, respectively (n = 6).

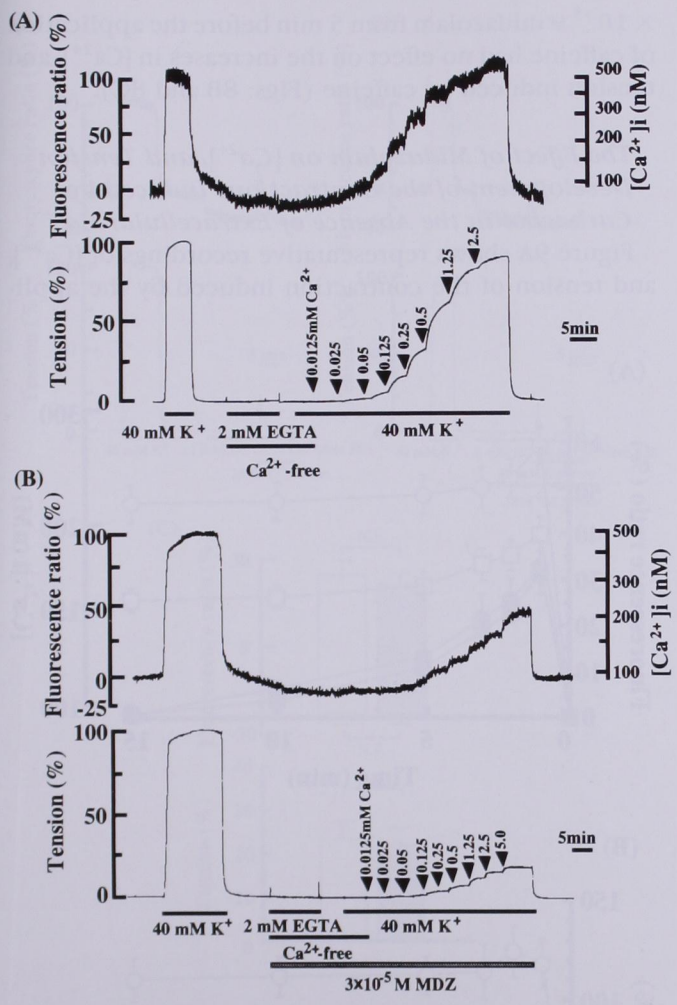

Fig. 4. Effect of 3×10^{-5} M midazolam on the increases in $[Ca^{2+}]_i$ and tension induced by cumulative application of extracellular Ca^{2+} (0.0125–2.5 or 5 mM) during 40 mM K^+ depolarization. Representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution, without (*A*) and with (*B*) 3×10^{-5} M midazolam treatment.

The Effect of Midazolam on the $[Ca^{2+}]_i$-Tension Relationship

Figure 6 represents the $[Ca^{2+}]_i$-tension relationships during the contractions induced by cumulative applications of the extracellular Ca^{2+} during high K^+ depolarization, in the presence or absence of carbachol, with or without midazolam treatment, were evaluated from data in figures 4 and 5. The $[Ca^{2+}]_i$-tension relationship of the contractions induced by the increases in the extracellular Ca^{2+} concentration during high K^+ depolarization in the presence of carbachol significantly shifted upward and left from that in the absence of carbachol. Treatment with 3×10^{-5} M midazolam had no significant effect on the $[Ca^{2+}]_i$-tension relationship both in the absence and presence of carbachol.

The Effects of the Pretreatment with Midazolam on the Dynamic Changes in $[Ca^{2+}]_i$ and Tension during the Contractions Induced by Carbachol

As shown in figure 7, increases in both $[Ca^{2+}]_i$ and tension induced by 10^{-7} M carbachol were inhibited

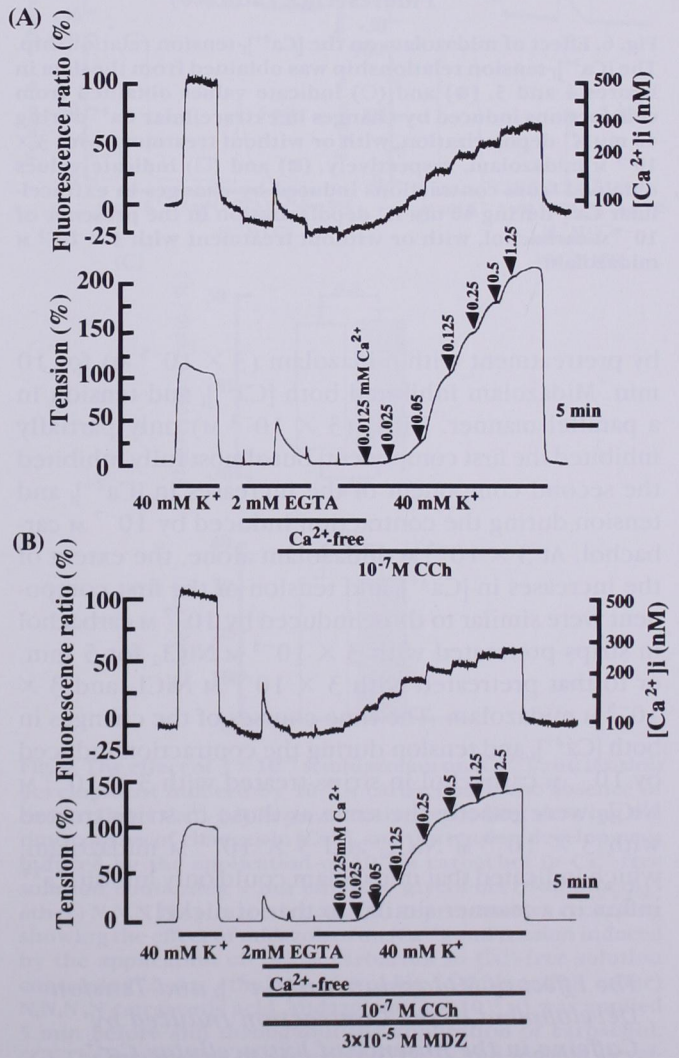

Fig. 5. Effect of 3×10^{-5} M midazolam on the increases in $[Ca^{2+}]_i$ and tension induced by cumulative application of extracellular Ca^{2+} (0.0125–1.25 or 2.5 mM) during 40 mM K^+ depolarization in the presence of 10^{-7} M carbachol. Representative recordings of changes in $[Ca^{2+}]_i$ and tension induced by the cumulative application of $CaCl_2$ in Ca^{2+}-free 40 mM K^+ solution in the presence of 10^{-7} M carbachol, without (*A*) and with (*B*) 3×10^{-5} M midazolam treatment.

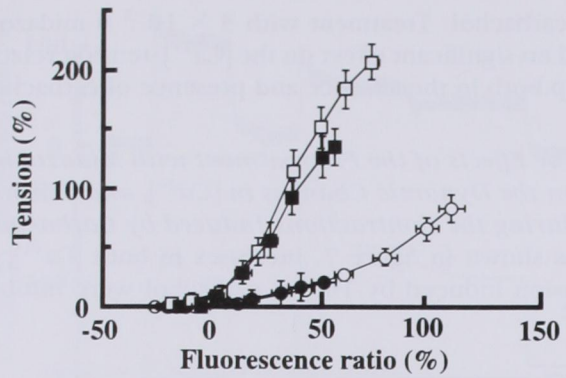

Fig. 6. Effect of midazolam on the $[Ca^{2+}]_i$-tension relationship. The $[Ca^{2+}]_i$-tension relationship was obtained from the data in figures 4 and 5. (●) and (○) indicate values obtained from contractions induced by changes in extracellular Ca^{2+} during 40 mM K^+ depolarization, with or without treatment with 3×10^{-5} M midazolam, respectively. (■) and (□) indicate values obtained from contractions induced by changes in extracellular Ca^{2+} during 40 mM K^+ depolarization in the presence of 10^{-7} M carbachol, with or without treatment with 3×10^{-5} M midazolam.

by pretreatment with midazolam (3×10^{-5} M) for 10 min. Midazolam inhibited both $[Ca^{2+}]_i$ and tension in a parallel manner. $NiCl_2$ (3×10^{-3} M) only partially inhibited the first component, but almost fully inhibited the second component of the increases in $[Ca^{2+}]_i$ and tension during the contraction induced by 10^{-7} M carbachol. At 3×10^{-5} M midazolam alone, the extent of the increases in $[Ca^{2+}]_i$ and tension of the first component were similar to those induced by 10^{-7} M carbachol in strips pretreated with 3×10^{-3} M $NiCl_2$ for 5 min, or to that pretreated with 3×10^{-3} M $NiCl_2$ and 3×10^{-5} M midazolam. The time courses of the changes in both $[Ca^{2+}]_i$ and tension during the contraction induced by 10^{-7} M carbachol in strips treated with 3×10^{-3} M $NiCl_2$ were exactly the same as those in strips treated with 3×10^{-3} M $NiCl_2$ and 3×10^{-5} M midazolam, which indicated that midazolam could only inhibit Ca^{2+} influx in a manner similar to that of nickel.

The Effect of Midazolam on $[Ca^{2+}]_i$ and Tension Development of the Contraction Induced by Caffeine in the Absence of Extracellular Ca^{2+}

Figure 8A shows the representative recordings of $[Ca^{2+}]_i$ and tension of the contraction induced by the application of 20 mM caffeine in Ca^{2+}-free solution containing 2 mM EGTA. The application of 20 mM caffeine in the absence of extracellular Ca^{2+} caused a transient increase in $[Ca^{2+}]_i$ and tension. Treatment with 3×10^{-5} M midazolam from 5 min before the application of caffeine had no effect on the increases in $[Ca^{2+}]_i$ and tension induced by caffeine (Figs. 8B and 8C).

The Effect of Midazolam on $[Ca^{2+}]_i$ and Tension Development of the Contractions Induced by Carbachol in the Absence of Extracellular Ca^{2+}

Figure 9A shows representative recordings of $[Ca^{2+}]_i$ and tension of the contraction induced by the appli-

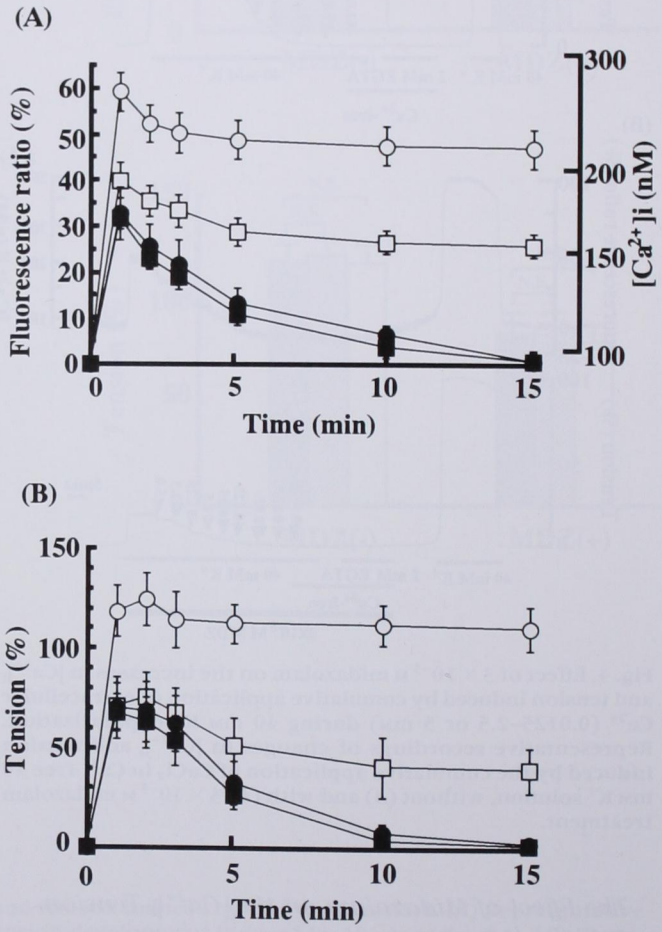

Fig. 7. The effect of pretreatment with midazolam on changes in $[Ca^{2+}]_i$ and the tension induced by 10^{-7} M carbachol. The time courses of changes in (*A*) $[Ca^{2+}]_i$ and (*B*) tension development induced by 10^{-7} M carbachol in strips with (□) or without (control) (○) pretreatment with 3×10^{-5} M midazolam are shown. The application of midazolam was started 10 min prior to that of carbachol. The abscissa scales indicate the time (in min) after the application of carbachol. Time courses of changes in $[Ca^{2+}]_i$ and tension induced by 10^{-7} M carbachol in the strips with (■) or without (control) (●) pretreatment with 3×10^{-5} M midazolam in the presence of 3×10^{-3} M $NiCl_2$ are also shown. $NiCl_2$ was applied 15 min before the application of carbachol. The data represent the mean of 7 different measurements; the vertical bars show the standard error.

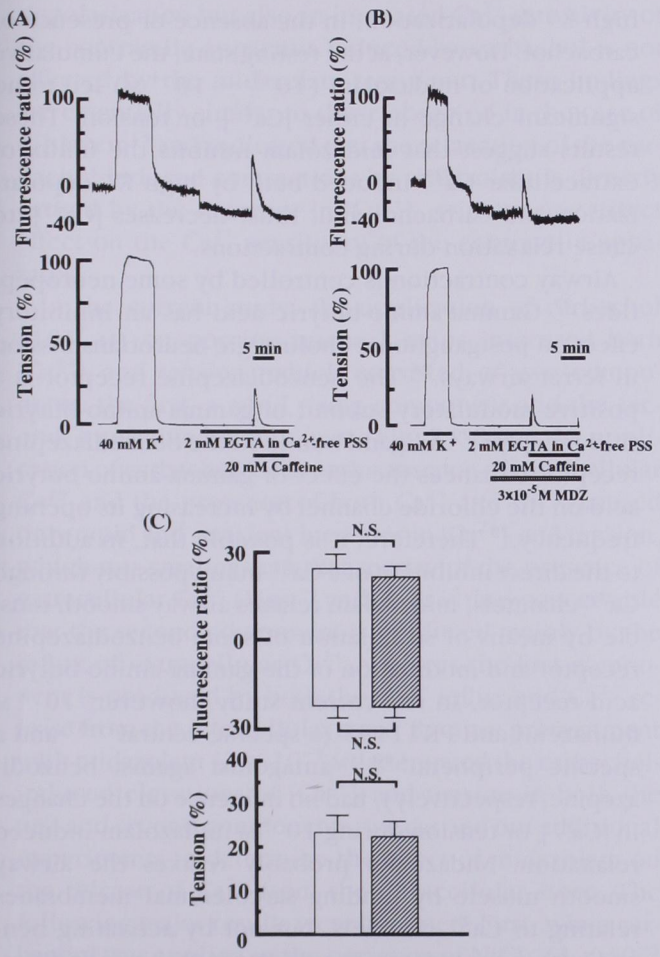

Fig. 8. The effect of 3×10^{-5} M midazolam on $[Ca^{2+}]_i$ and tension development induced by 20 mM caffeine in the absence of extracellular Ca^{2+}. (*A*) Representative recording showing the time courses of changes in $[Ca^{2+}]_i$ and tension development induced by the applications of 20 mM caffeine in Ca^{2+}-free solution containing 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. (*B*) Representative recording showing the effect of midazolam on $[Ca^{2+}]_i$ and tension development induced by the application of 20 mM caffeine in a Ca^{2+}-free solution containing 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. Midazolam (3×10^{-5} M) was applied 5 min before and throughout the application of caffeine. (*C*) Summaries of the changes in $[Ca^{2+}]_i$ and tension development in response to applications of caffeine, without (open columns) and with (hatched columns) 3×10^{-5} M midazolam treatment on the application of caffeine. The bottoms and the tops of each column indicate the $[Ca^{2+}]_i$ and tension just before the caffeine application and the peak levels obtained by the caffeine application, respectively. The vertical bars at the bottom and the top of each column show the standard error (n = 5).

cation of 10^{-7} M carbachol in Ca^{2+}-free solution containing 2 mM EGTA. The application of 10^{-7} M carbachol in the absence of extracellular Ca^{2+} caused a transient increase in $[Ca^{2+}]_i$ and tension. Treatment with

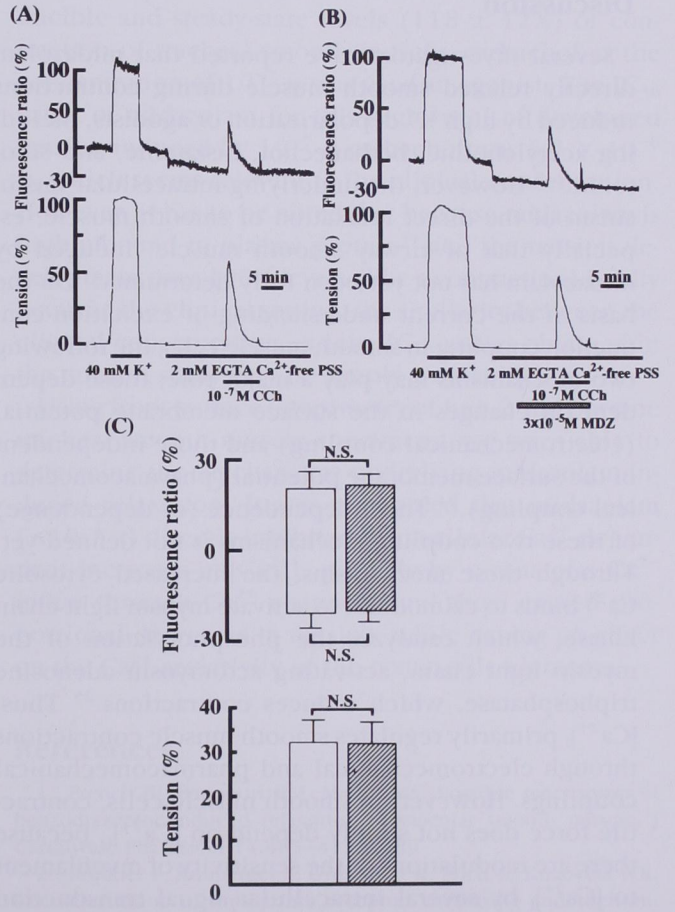

Fig. 9. The effect of 3×10^{-5} M midazolam on $[Ca^{2+}]_i$ and tension development induced by 10^{-7} M carbachol in the absence of extracellular Ca^{2+}. (*A*) Representative recording showing the time courses of changes in $[Ca^{2+}]_i$ and the tension development induced by the application of 10^{-7} M carbachol in Ca^{2+}-free solution containing 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. (*B*) Representative recording showing the effect of midazolam on $[Ca^{2+}]_i$ and tension induced by the application of 10^{-7} M carbachol in Ca^{2+}-free solution containing 2 mM ethylene glycol-bis (β-aminoethyl ether) N,N,N′,N′-tetraacetic acid. Midazolam (3×10^{-5} M) was applied 5 min before and throughout the application of carbachol. (*C*) The summaries of changes in $[Ca^{2+}]_i$ and tension development in response to applications of carbachol, without (open columns) and with (hatched columns) 3×10^{-5} M midazolam treatment on the application of carbachol. The bottoms and the tops of each column indicate the $[Ca^{2+}]_i$ and tension just before the carbachol-application and the peak levels obtained by the carbachol-application, respectively. The vertical bars at the bottom and the top of each column show the standard error (n = 5).

3×10^{-5} M midazolam from 5 min before the application of carbachol had no effect on the increases in $[Ca^{2+}]_i$ and tension induced by carbachol (Figs. 9B and 9C).

Discussion

Several investigators have reported that midazolam directly relaxed smooth muscle during contractions induced by high K^+ depolarization or agonists, including acetylcholine, bethanechol, histamine, and serotonin.[1–7] However, the underlying intracellular mechanism of the direct relaxation of smooth muscle, especially that of airway smooth muscle, induced by midazolam has not yet been fully determined. On the basis of the current understanding of excitation-contraction coupling in smooth muscle cells, the following two mechanisms may play a major role: those dependent on changes in the surface membrane potential (electromechanical coupling) and those independent of the surface membrane potential (pharmacomechanical coupling).[24] The independence (or dependence) of these two coupling mechanisms is not defined yet. Through these mechanisms, the increased cytosolic Ca^{2+} binds to calmodulin to activate myosin light chain kinase, which catalyzes the phosphorylation of the myosin light chain, activating actomyosin adenosine triphosphatase, which induces contractions.[25] Thus, $[Ca^{2+}]_i$ primarily regulates smooth muscle contractions through electromechanical and pharmacomechanical couplings. However, in smooth muscle cells, contractile force does not simply depend on $[Ca^{2+}]_i$, because there are modulations of the sensitivity of myofilament to $[Ca^{2+}]_i$ by several intracellular signal transduction systems; and this mechanism also may be included in pharmacomechanical coupling.[26] To clarify the mechanism of the midazolam-induced relaxation of airway smooth muscle, we determined the effect of midazolam on electromechanical and pharmacomechanical coupling, namely, on Ca^{2+} influx through the voltage-operated or receptor-operated Ca^{2+} channels, on Ca^{2+} release from the intracellular store, and on the Ca^{2+} sensitivity of the contractile apparatus.

In the current study at the steady state of both high K^+ depolarization-induced and carbachol-induced contractions, cumulative applications of midazolam ($10^{-7} \sim 10^{-4}$ M) caused a concentration-dependent decrease in $[Ca^{2+}]_i$ and tension. Treatment with 3×10^{-5} M midazolam inhibited the extracellularly applied Ca^{2+}-induced increases in $[Ca^{2+}]_i$ and tension during high K^+ depolarization, in the absence or presence of carbachol. However, at the resting state, the cumulative application of midazolam ($10^{-7} \sim 10^{-4}$ M) led to no significant change in either $[Ca^{2+}]_i$ or tension. These results suggest that midazolam inhibits the influx of extracellular Ca^{2+} induced both by high K^+ depolarization and carbachol and, thus, decreases $[Ca^{2+}]_i$ to cause relaxation during contractions.

Airway contraction is controlled by some neuropeptides.[27] Gamma-amino-butyric acid has an inhibitory effect on postganglionic cholinergic neurotransmission in ferret airways.[28] The benzodiazepine receptor is a positive modulatory subunit of gamma-amino-butyric acid receptor.[29] A ligand bound to the benzodiazepine receptor enhances the effect of gamma-amino-butyric acid on the chloride channel by increasing its opening frequency.[29] Therefore, it is possible that, in addition to the direct inhibition of a Ca^{2+} influx possibly through Ca^{2+} channels, midazolam relaxes airway smooth muscle by means of stimulation of some benzodiazepine receptor and modulation of the gamma-amino-butyric acid receptor. In the current study, however, 10^{-5} M flumazenil and PK11195 (a specific central[14–17] and a specific peripheral[1,18,19] antagonist against benzodiazepine, respectively), had no influence on the changes in $[Ca^{2+}]_i$ or tension during 10^{-5} M midazolam-induced relaxation. Midazolam probably relaxes the airway smooth muscle by binding sarcolemmal membranes relating to Ca^{2+} channels, but not by activating benzodiazepine receptors. In agreement with our findings, flumazenil and PK11195 have been reported to have no effect on midazolam-induced relaxation of airway smooth muscle.[5,7] The concentration of flumazenil (10^{-5} M) used in the current study is higher than the estimated levels of plasma concentrations in clinical use.[14–17]

In the presence of carbachol, the $[Ca^{2+}]_i$-tension relation-curve of the contractions induced by the increases in extracellular Ca^{2+} concentration during high K^+-depolarization, shifted markedly upward and left from that obtained in the absence of carbachol. In other words, at a given $[Ca^{2+}]_i$ level, the tension development in the presence of carbachol was much greater than that in the absence of carbachol, indicating that the Ca^{2+} sensitivity of the contractile apparatus is increased by carbachol. Similar findings, that muscarinic agonists increase myofilament Ca^{2+} sensitivity in airway smooth muscle, have been reported by others.[20,30–32] In addition, the current study showed that, not only the Ca^{2+} sensitivity during the contraction induced by high K^+-

depolarization but also an increased Ca^{2+} sensitivity of the contractile apparatus induced by carbachol is not affected by the midazolam treatment. These findings are essentially similar to those observed in the case of diltiazem[13] and indicated that the relaxation of the carbachol-induced contractions by midazolam is directly caused by the decrease in $[Ca^{2+}]_i$, without any direct effect on the Ca^{2+} sensitivity of the contractile apparatus.

In the current study, the application of carbachol (10^{-7} M) on porcine tracheal strips increased both $[Ca^{2+}]_i$ and tension, which consisted of two components: the first, a rapid rising component and the second, a sustained component (Fig. 7). Since the application of carbachol, both in the absence of extracellular Ca^{2+} and the presence of both Ca^{2+} and Ni^{2+}, caused only rapid and transient increases in $[Ca^{2+}]_i$ and tension, which was smaller than that seen with the presence of extracellular Ca^{2+} (Figs. 7 and 9), it is thus conceivable that the second component is produced mainly by the influx of extracellular Ca^{2+}, whereas the first component is produced by both the Ca^{2+} influx and Ca^{2+} release from the intracellular store. Because pretreatment with midazolam (3×10^{-5} M) attenuated the carbachol-induced elevations of $[Ca^{2+}]_i$ and tension in both the first and second components, we carried out additional experiments to determine the effect of midazolam on the release of Ca^{2+} from the intracellular store. The following major results were obtained: First, when carbachol was applied in the presence of $NiCl_2$ (3×10^{-3} M) to inhibit the Ca^{2+} influx from the extracellular spaces, midazolam (3×10^{-5} M) did not affect the elevation of $[Ca^{2+}]_i$ and tension of the first component at all. Second, the effect of midazolam on the Ca^{2+} release by two distinct stimulants, caffeine and carbachol, in the absence of extracellular Ca^{2+} was examined. It is well established that caffeine causes Ca^{2+} release by facilitating the Ca^{2+}-induced Ca^{2+} release mechanism, whereas carbachol causes Ca^{2+} release by pharmacomechanical coupling mediated by inositol 1,4,5,-trisphosphate.[33] In the absence of extracellular Ca^{2+}, 3×10^{-5} M midazolam was not found to have any inhibiting effect on the increases in $[Ca^{2+}]_i$ and tension induced by either caffeine or carbachol. We found that only an extremely high concentration of midazolam (3×10^{-4} M) inhibited the increases in $[Ca^{2+}]_i$ and tension due to the release of Ca^{2+} from the intracellular store induced by either caffeine or carbachol. However, the effects of midazolam, at a concentration higher than this level was not reversible (data not shown). These observations indicate that the clinical concentrations of midazolam do not affect Ca^{2+} release from the intracellular stores.

The blood concentration of benzodiazepines used clinically is 100 ~ 200 μg/ml, *i.e.*, approximately 10^{-7} ~ 10^{-6} M.[34–37] In the current study, to observe the definite relaxation with midazolam during the reproducible and steady-state levels (118 ± 12%) of contraction of tracheal smooth muscle, carbachol at the concentration of 10^{-7} M was used an agonist. The IC_{50} value of midazolam for this high level of developed tension induced by 10^{-7} M carbachol was 6.3×10^{-6} M, which seems relevant to the clinical concentration. However, it has to be noted that because midazolam is highly bound to plasma protein[34] and the tension development over 100% levels (like a contraction) hardly occur in the clinical setting, the similarity between the clinical concentration and the IC_{50} values obtained in the current study may be simply a coincidence.

Using front-surface fluorometry of fura-2 and porcine tracheal smooth muscle specimens, we were able to determine the mechanisms underlying midazolam-induced relaxation. It was concluded that midazolam ($\leq 10^{-4}$ M) directly inhibited extracellular Ca^{2+}-dependent increases in $[Ca^{2+}]_i$, possibly by means of a Ca^{2+} influx through Ca^{2+} channels, and thus, caused proportional decreases in tension, while having no effect on the Ca^{2+} sensitivity of the contractile elements.

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