Benzodiazepines Differentially Inhibit Phenylephrine-induced Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

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Background: Modulation of intracellular free calcium is a critical determinant of vasomotor tone. The authors investigated the effects of three benzodiazepines on α-adrenergic–induced oscillations in intracellular free calcium in individual pulmonary artery smooth muscle cells.

Methods: Pulmonary artery smooth muscle cells were cultured from explants of canine intrapulmonary artery. Fura-2–loaded pulmonary artery smooth muscle cells were continuously superfused with phenylephrine (10 μM) at 37°C on the stage of an inverted fluorescence microscope. Intracellular free calcium was measured using a dual wavelength spectrofluorometer. After establishment of steady-state intracellular free calcium oscillations induced by phenylephrine, lorazepam, diazepam, or midazolam was added to the superfusate. The amplitude and frequency of the intracellular free calcium oscillations were compared before and after addition of each agent.

Results: Resting mean ± SEM values of intracellular free calcium were 68 ± 8 nM. Phenylephrine stimulated dose-dependent oscillations in intracellular free calcium, which reached a peak concentration of 676 ± 35 nM and a frequency of 1.08 ± 0.1 transients/min. Addition of lorazepam (1 μM) inhibited ($P < 0.05$) the amplitude (591 ± 32 nM) but not the frequency (0.97 ± 0.1 transients/min) of the oscillations. Conversely, diazepam (1 μM) decreased ($P < 0.05$) the frequency (0.79 ± 0.1 transients/min) but not the amplitude (663 ± 37 nM) of the oscillations. These effects were dose-dependent. In contrast, midazolam (1–30 μM) had no effect on the amplitude or frequency of intracellular free calcium oscillations. At concentrations higher than 100 μM, however, all three benzodiazepines inhibited both the amplitude and frequency of the intracellular free calcium oscillations.

Conclusion: Lorazepam and diazepam but not midazolam exerted differential inhibitory effects on phenylephrine-induced intracellular free calcium oscillations. Benzodiazepines may alter the pulmonary vascular response to sympathetic α-adrenoreceptor activation by direct inhibition of intracellular free calcium signaling in pulmonary artery smooth muscle cells. (Key words: Canine; intracellular Ca^{2+}; sympathetic α-adrenoreceptors.)

BENZODIAZEPINES are widely used in anesthetic practice as a premedicant or a sedative–amnesic or induction agent. Hemodynamic alterations induced by benzodiazepines are mediated, in part, through inhibitory effects on the sympathetic nervous system.[1-3] Benzodiazepines, however, also may exert direct effects on smooth muscle.[4-6] Inhibition of sympathetic control of vasomotor tone could be deleterious in certain clinical situations, such as hypovolemia and heart failure, when sustained adrenergic activity is needed to maintain cardiovascular homeostasis.[7]

In general, intravenous anesthetic agents have been shown to exert a pulmonary vasodilator influence and attenuate agonist-induced increases in pulmonary vasomotor tone.[6-10] Changes in pulmonary vascular smooth muscle intracellular calcium ([Ca^{2+}]$_i$) concentration are important in the short-term modulation of vasomotor tone in response to neural, humoral, or local activation. Therefore, inhibitory effects of benzodiazepines could be due to alterations in pulmonary vascular smooth muscle [Ca^{2+}]$_i$ signaling in response to agonist activation. We recently reported that sympathetic α-adrenoreceptor stimulation of individual canine pulmonary artery smooth muscle cells (PASMCs) induces oscillations in [Ca^{2+}]$_i$.[11] We also reported that several intravenous anesthetic agents (thiopental, ketamine, propofol) differentially attenuate the amplitude or frequency of [Ca^{2+}]$_i$ oscillations induced by α-adrenoreceptor activa-

tion.[12] Changes in the amplitude or frequency of $[Ca^{2+}]_i$ oscillations may play a role in the short-term modulation of pulmonary vasomotor tone in response to agonist activation. The effects of benzodiazepines on agonist-induced $[Ca^{2+}]_i$ signaling in PASMCs have not been examined. The goal of the current study was to investigate the effects of lorazepam, diazepam, and midazolam on sympathetic α-adrenergic–induced $[Ca^{2+}]_i$ signaling in individual PASMCs. We tested the hypothesis that benzodiazepines would reduce the amplitude or frequency of $[Ca^{2+}]_i$ oscillations in response to the α-adrenoreceptor agonist, phenylephrine.

Materials and Methods

Animals

Pulmonary arteries were isolated from adult male mongrel dogs. The technique of euthanasia was approved by the Institutional Animal Care and Use Committee. All procedures were performed aseptically using general anesthesia (fentanyl, 15 μg/kg; pentobarbital sodium, 30 mg/kg, given intravenously), an endotracheal tube, and positive pressure ventilation. A catheter was placed in the right femoral artery, the mobilizable blood volume was removed, and 30 ml of saturated KCl was administered intravenously. A left thoracotomy was performed through the fifth intercostal space. The heart and lungs were removed *en bloc,* and the pulmonary arteries were isolated and dissected in a laminar flow hood under sterile conditions.

Cell Culture

Primary cultures of PASMCs were obtained from segmental and subsegmental intralobar branches of pulmonary artery (the third and fourth generation of branches from the main pulmonary artery) having diameters <4 mm. Explant cultures were prepared according to the method of Campbell and Campbell[13] with minor modifications. The endothelium was removed by gentle rubbing with a sterile cotton swab. The tunica adventitia was carefully removed together with the most superficial part of the tunica media. The remaining tunica media was cut into 2-mm^2 pieces that were explanted in 25-cm^2 culture flasks. The explants were nourished by D-MEM/F-12 medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum and 1% antibiotic–antimycotic mixture solution (10,000 U/ml penicillin, 10,000 μg/ml streptomycin, and 25 μg/ml of amphoteri-

cin B) and kept in a humidified atmosphere of 5% CO_2:95% air at 37°C. Pulmonary artery smooth muscle cells began to proliferate from explants after 7 days in culture. Cells were allowed to proliferate for an additional 7–10 days until subconfluence was achieved. Cells were then subcultured nonenzymatically to 35-mm culture dishes specially designed for fluorescence microscopy (Bioptechs Inc., $\triangle$T system, Butler, PA). Pulmonary artery smooth muscle cells were used for experimentation within 72 h after subculture. Cells from the first and second passage were used for experiments. These cells were routinely identified as smooth muscle cells using a fluorescein-labeled antibody directed at smooth muscle α actin (Sigma Chemical Co., St. Louis, MO).

Fura-2 Loading Procedure

Twenty-four hours before experimentation, the culture medium containing 10% fetal bovine serum was replaced with serum-free medium to arrest cell growth, to allow for establishment of steady-state cellular events independent of cell division, and to prevent a false estimate of $[Ca^{2+}]_i$ because of binding of available dye to serum proteins in the medium.[14] Pulmonary artery smooth muscle cells were washed twice in loading buffer, which contained (in mM) 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 11 glucose, 1.8 $CaCl_2$, 25 HEPES, and 0.2% bovine serum albumin at pH 7.4 adjusted with NaOH. Pulmonary artery smooth muscle cells were then incubated in loading buffer containing 2 μM fura-2/AM, the acetoxymethyl derivative of fura-2 (Molecular Probes, Eugene, OR), at ambient temperature for 30 min. After the 30-min loading period, the cells were washed twice in loading buffer and incubated at ambient temperature for an additional 20 min before study. This provided sufficient time to wash away any extracellular fura-2/AM and for intracellular esterases to cleave fura-2/AM into the active fura-2.[15]

Determination of Intracellular Free Calcium

Culture dishes containing fura-2–loaded PASMCs were placed in a temperature-regulated (37°C) chamber (Bioptechs, Inc., Butler, PA) mounted on the stage of an Olympus IX-70 inverted fluorescence microscope (Olympus America Inc., Lake Success, NY). Fluorescence measurements were performed on individual PASMCs on a cultured monolayer using a dual wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, So. Brunswick, NJ) at excita-

tion wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The volume of the chamber was 1.5 ml. The cells were superfused continuously at 1 ml/min with Krebs–Ringer buffer, which contained (in mM) 125 NaCl, 5 KCl, 1.2 $MgSO_4$, 11 glucose, 2.5 $CaCl_2$, and 25 HEPES at pH 7.4 adjusted with NaOH. The temperature of all solutions was maintained at 37°C in a water bath. Just before data acquisition, background fluorescence (*i.e.,* fluorescence between cells) was measured and subtracted automatically from the subsequent experimental measurements. Fura-2 fluorescence signals (340, 380, and 340/380 ratio) originating from single PASMCs were continuously monitored at a sampling frequency of 25 Hz and were collected using a software package from Photon Technology International (Felix™).

Titration of Fura-2

Estimates of $[Ca^{2+}]_i$ were made by comparing the cellular fluorescence ratio with ratios acquired using fura-2 (free acid) in buffers containing known Ca^{2+} concentrations. $[Ca^{2+}]_i$ was then calculated as described by Grynkiewicz *et al.*[16]

Experimental Protocol

Pulmonary artery smooth muscle cells were pretreated with the β-adrenoreceptor antagonist propranolol (5 μM) to eliminate any β-agonist effect of phenylephrine. The effects of increasing doses of phenylephrine on the amplitude and frequency of $[Ca^{2+}]_i$ were first assessed. In subsequent experiments, after establishment of steady-state $[Ca^{2+}]_i$ oscillations induced by 10 μM phenylephrine, lorazepam, diazepam, or midazolam was added to the superfusate in the continued presence of phenylephrine. Any given PASMC was exposed to only one agent. Lorazepam (2 mg/ml stock) was administered at concentrations ranging from 0.3–100.0 μM, diazepam (5 mg/ml stock) at 3–300 μM, and midazolam (5 mg/ml stock) at 1–100 μM with 5-min intervals between doses. The changes in solution were accomplished by rapidly aspirating the buffer in the dish and transiently increasing the flow rate to 10 ml/min. In a separate series of experiments, the vehicle for diazepam (1 ml contains 0.4 ml propylene glycol, 0.1 ml alcohol, 0.015 ml benzyl alcohol and water) or lorazepam (1 ml contains 0.18 ml polyethylene glycol 400 in propylene glycol with 0.02 ml benzyl alcohol) was added to the superfusate as a vehicle control at a concentration corresponding to 300 μM.

Data Analysis

The amplitude and frequency of phenylephrine-induced $[Ca^{2+}]_i$ oscillations were measured in individual PASMCs. The amplitude was calculated by averaging the peak ratio obtained for 4–5 oscillations before and after each intervention. The change in the 340/380 fluorescence ratio was then calculated by subtracting the resting ratio value (baseline). The frequency of oscillations was calculated by averaging the time interval between the oscillation peaks and is reported as the number of oscillations observed per minute. Changes in the amplitude and frequency of the 340/380 fluorescence ratio in response to the benzodiazepines are expressed as percent of control, in which control equals the amplitude and frequency of oscillations induced by 10 μM phenylephrine (without benzodiazepines). Data are expressed as means $\pm$ SEM. Statistical analysis was performed using repeated-measures analysis of variance followed by Bonferroni/Dunn *post hoc* testing. Differences were considered statistically significant at $P < 0.05$.

Reagents

Phenylephrine and propranolol were purchased from Sigma Chemical Co. Lorazepam (Ativan®) was obtained from Wyeth Laboratories Inc. (Philadelphia, PA), diazepam (Valium®) from ESI Pharmaceuticals (Cherry Hill, NJ), and midazolam (Versed®) from Roche Laboratories (Nutley, NJ). Vehicle for diazepam and lorazepam was obtained from The Cleveland Clinic Pharmacy (Cleveland, OH).

Results

Characteristics of Phenylephrine-evoked Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

A typical trace depicting the effect of phenylephrine (10 μM) on $[Ca^{2+}]_i$ in a single PASMC is shown in figure 1. Resting values of $[Ca^{2+}]_i$ were 68 $\pm$ 8 nM. Peak $[Ca^{2+}]_i$ achieved with 10 μM phenylephrine was 676 $\pm$ 35 nM at a frequency of 1.08 $\pm$ 0.1 transients/min. As previously noted,[11,12] tachyphylaxis to phenylephrine was never observed, and oscillations maintained a constant amplitude and frequency for >30 min. Summarized data depicting the dose-dependent effects of phenylephrine on the amplitude and frequency of $[Ca^{2+}]_i$ oscillations are also shown in figure 1 (n = 19 cells). The lowest concentration of phenylephrine that induced $[Ca^{2+}]_i$ oscillations was 3 × 10^{-8} M, achieving a peak $[Ca^{2+}]_i$ concentration of 198 $\pm$
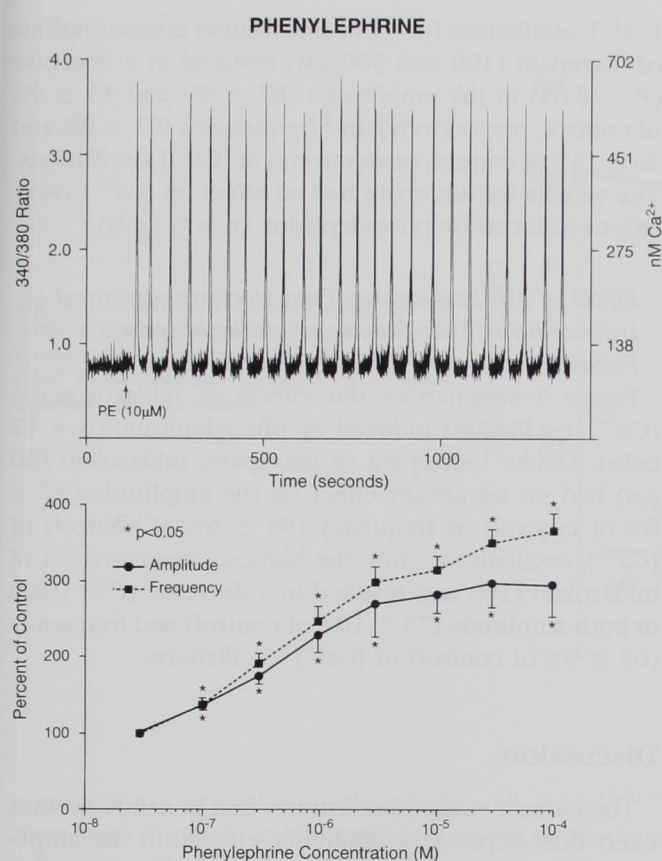
PHENYLEPHRINE

Fig. 1. *(Top)* A representative trace demonstrating the effect of phenylephrine (PE; 10 μM) on intracellular free calcium ($[Ca^{2+}]_i$) concentration in a single pulmonary artery smooth muscle cell (PASMC). Estimates of $[Ca^{2+}]_i$ were made by comparing the cellular fluorescence ratio with ratios acquired using fura-2 (free acid) in buffers containing known concentrations of Ca^{2+}. Values for $[Ca^{2+}]_i$ were calculated as previously described[16] and are presented on the right *y* axis. *(Bottom)* Summarized data depicting the dose-dependent increases in both the amplitude and the frequency of $[Ca^{2+}]_i$ oscillations (340/380 ratio) induced by phenylephrine (n = 19 cells).

19 nM at a frequency of 0.31 ± 0.1 transients/min. These results were defined as the control response, and the amplitude and frequency were normalized to 100%. The frequency and amplitude of phenylephrine-induced $[Ca^{2+}]_i$ oscillations were similar in first- and second-passage PASMCs.

Effects of Lorazepam on Phenylephrine-induced Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

Figure 2 shows a typical trace demonstrating the dose-dependent inhibitory effect of lorazepam on

peak $[Ca^{2+}]_i$ in a single PASMC. Lorazepam (1 μM) exerted a significant inhibitory effect ($P < 0.05$) on the amplitude (87 ± 3% of control) but not the frequency (96 ± 4% of control) of $[Ca^{2+}]_i$ oscillations. The inhibitory effect of lorazepam on the amplitude of $[Ca^{2+}]_i$ oscillations was dose-dependent. Figure 2 summarizes the dose-dependent effects of lorazepam on both the amplitude and frequency of phenylephrine-induced $[Ca^{2+}]_i$ oscillations (n = 13 cells). Only the highest concentration of lorazepam (100 μM) decreased ($P < 0.05$) the frequency (77 ± 13% of control) of $[Ca^{2+}]_i$ oscillations. The vehicle for
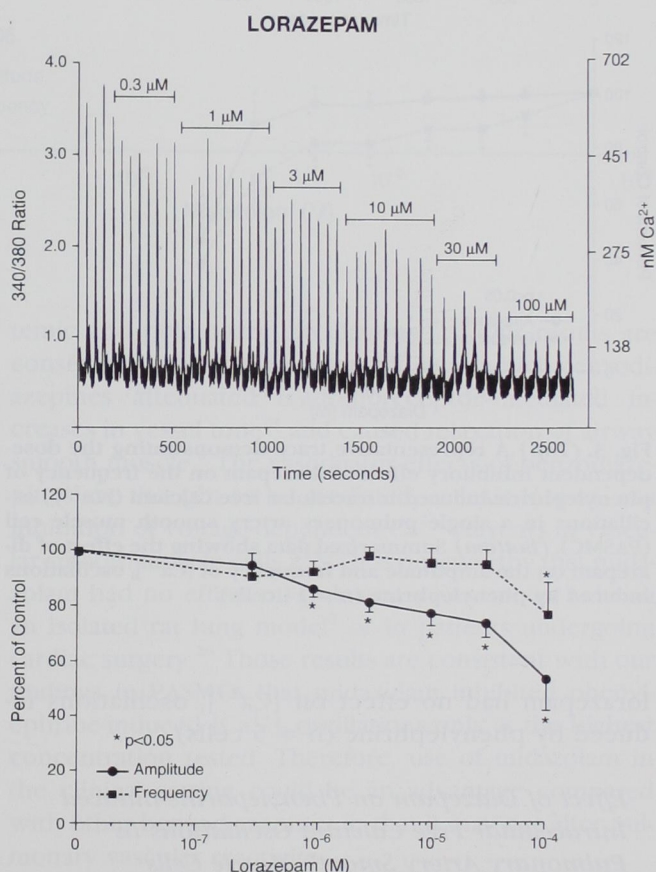
LORAZEPAM

Fig. 2. *(Top)* A representative trace demonstrating the dose-dependent inhibitory effect of lorazepam on the amplitude of phenylephrine-induced intracellular free calcium ($[Ca^{2+}]_i$) oscillations in a single pulmonary artery smooth muscle cell (PASMC). Increasing concentrations of lorazepam were added to the superfusion buffer as indicated in the continuous presence of phenylephrine (10 μM). *(Bottom)* Summarized data showing the effects of lorazepam on the amplitude and frequency of $[Ca^{2+}]_i$ oscillations induced by phenylephrine (n = 13 cells).
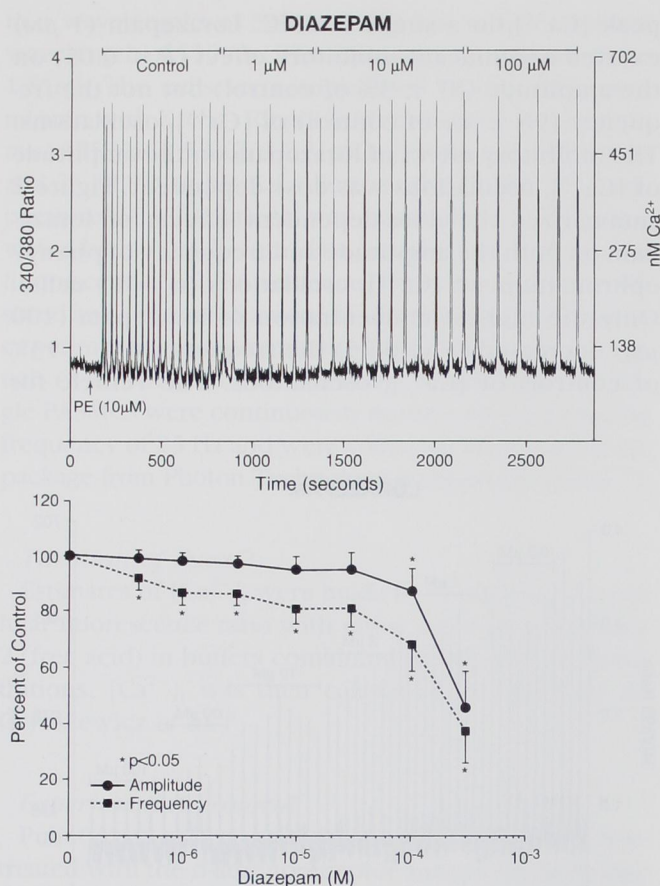
Fig. 3. *(Top)* A representative trace demonstrating the dose-dependent inhibitory effect of diazepam on the frequency of phenylephrine-induced intracellular free calcium ([Ca^{2+}]$_i$) oscillations in a single pulmonary artery smooth muscle cell (PASMC). *(Bottom)* Summarized data showing the effect of diazepam on the amplitude and frequency of [Ca^{2+}]$_i$ oscillations induced by phenylephrine (n = 14 cells).

lorazepam had no effect on [Ca^{2+}]$_i$ oscillations induced by phenylephrine (n = 5 cells).

Effect of Diazepam on Phenylephrine-induced Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

Figure 3 shows a typical trace demonstrating the dose-dependent inhibitory effect of diazepam on the frequency of [Ca^{2+}]$_i$ oscillations in a single PASMC. Figure 3 also summarizes the dose-dependent inhibitory effect of diazepam on the amplitude and frequency of [Ca^{2+}]$_i$ oscillations induced by phenylephrine. Diazepam (0.3 μM) reduced ($P < 0.05$) the frequency (86 ± 4% of control) but not the amplitude (98 ± 2% of control) of

[Ca^{2+}]$_i$ oscillations (n = 14 cells). Higher concentrations of diazepam (100 and 300 μM) resulted in a decrease ($P < 0.05$) in the amplitudes (87 ± 5% and 45 ± 9% of control, respectively) and frequencies (87 ± 8% and 46 ± 13% of control, respectively) of [Ca^{2+}]$_i$ oscillations. The vehicle for diazepam had no effect on [Ca^{2+}]$_i$ oscillations induced by phenylephrine (n = 5 cells).

Effect of Midazolam on Phenylephrine-induced Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

Figure 4 summarizes the effects of midazolam on [Ca^{2+}]$_i$ oscillations induced by phenylephrine (n = 12 cells). Unlike lorazepam or diazepam, midazolam (30 μM) had no significant effect on the amplitude (97 ± 6% of control) or frequency (94 ± 6% of control) of [Ca^{2+}]$_i$ oscillations. Only the highest concentration of midazolam (100 μM) resulted in a decrease ($P < 0.05$) in both amplitude (75 ± 10% of control) and frequency (69 ± 9% of control) of [Ca^{2+}]$_i$ oscillations.

Discussion

The current study demonstrates that benzodiazepines exert dose-dependent inhibitory effects on the amplitude and frequency of phenylephrine-induced [Ca^{2+}]$_i$ oscillations in individual PASMCs. Both lorazepam and diazepam inhibited the phenylephrine-induced [Ca^{2+}]$_i$ oscillations in a differential manner. Lorazepam inhibited the amplitude of the [Ca^{2+}]$_i$ oscillations, whereas diazepam inhibited the frequency of the [Ca^{2+}]$_i$ oscillations. In contrast, the effects of midazolam were only apparent at very high concentrations.

Signal Transduction Pathway for Phenylephrine-induced Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

Activation of sympathetic α-adrenoreceptors causes vascular smooth muscle contraction by increasing [Ca^{2+}]$_i$.[17] The α-adrenoreceptor is coupled *via* a G protein to phospholipase C, which hydrolyzes polyphosphatidylinositol 4,5-bisphosphate. The breakdown of polyphosphatidylinositol 4,5-bisphosphate leads to the generation of cytosolic inositol trisphosphate and diacylglycerol.[18] Inositol trisphosphate stimulates the release of Ca^{2+} from intracellular stores initiating contraction, whereas diacylglycerol, which stimulates protein kinase C,[19] is involved in the maintenance of sustained
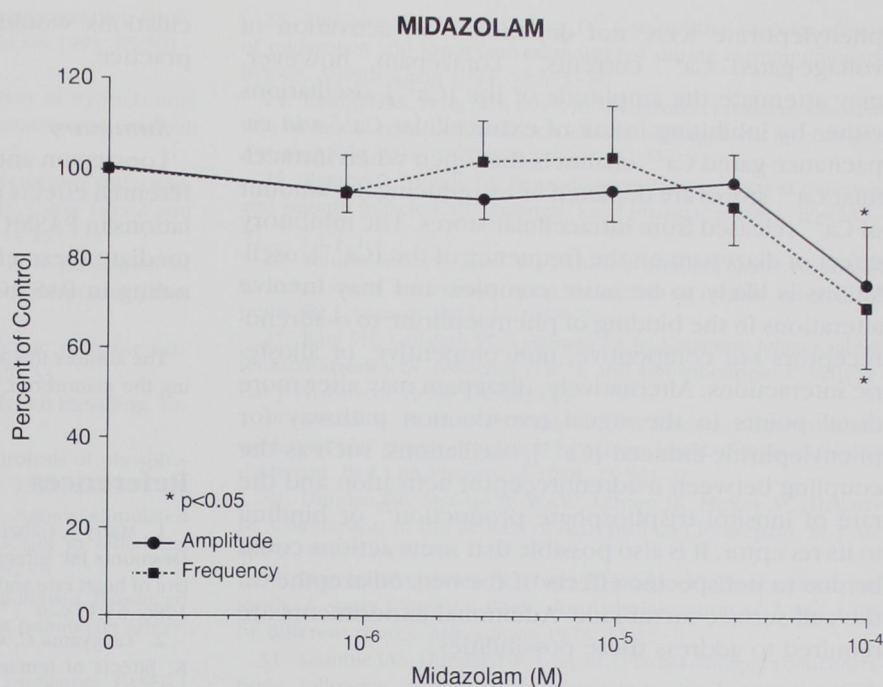
MIDAZOLAM

Fig. 4. Summarized data showing the effects of midazolam on the amplitude and frequency of intracellular free calcium ([Ca^{2+}]$_i$) oscillations induced by phenylephrine (n = 12 cells).

contractions. Several smooth muscle cell types[11,20,21] have been shown to exhibit agonist-induced oscillations in [Ca^{2+}]$_i$ with dose-dependent amplitudes and frequencies. This suggests that [Ca^{2+}]$_i$ may exert effects on smooth muscle tone through a frequency-dependent mechanism. We recently demonstrated that cultured and freshly dispersed individual PASMCs oscillate [Ca^{2+}]$_i$ in response to α-adrenoreceptor activation with phenylephrine.[11] These [Ca^{2+}]$_i$ oscillations were dependent on the presence of extracellular Ca^{2+} but did not require activation of voltage-gated Ca^{2+} channels. In addition, the [Ca^{2+}]$_i$ oscillations required activation of phospholipase C and involved the release of Ca^{2+} from caffeine-sensitive intracellular stores.[11] The frequency of [Ca^{2+}]$_i$ oscillations may represent a digitization of the Ca^{2+} signal, allowing frequency-dependent control of the contractile response for "fine tuning" vessel tone.[22]

Differential Inhibitory Effects of Lorazepam and Diazepam on Phenylephrine-induced Intracellular Free Calcium Oscillations in Pulmonary Artery Smooth Muscle Cells

In vivo studies suggest that concentrations of benzodiazepines used to induce surgical anesthesia can produce significant hemodynamic alterations in the sys-

temic and pulmonary circulations.[10,23] Our results are consistent with other *in vitro* studies in which benzodiazepines attenuated α-adrenoreceptor–mediated increases in vessel tone[24] and caused relaxation of airway smooth muscle.[6] The vasodilator effects of benzodiazepines are thought to be mediated, at least in part, *via* inhibition of voltage-dependent Ca^{2+} currents in vascular smooth muscle cells.[4,24,25] It is noteworthy that midazolam had no effect on pulmonary vasomotor tone in an isolated rat lung model[9] or in patients undergoing cardiac surgery.[26] Those results are consistent with our findings in PASMCs that midazolam inhibited phenylephrine-induced [Ca^{2+}]$_i$ oscillations only at the highest concentration tested. Therefore, use of midazolam in the clinical setting could be an advantage compared with other benzodiazepines in that it may not alter pulmonary vascular reactivity.

In the intact blood vessel, the vasodilating effects of benzodiazepines could be due to inhibition of voltage-gated Ca^{2+} channels preventing Ca^{2+} entry in the smooth muscle cells. In the current study, however, it is unlikely that the inhibitory mechanism of action of lorazepam or diazepam involves an interaction with voltage-gated Ca^{2+} channels, because we previously demonstrated that the [Ca^{2+}]$_i$ oscillations induced by

phenylephrine were not dependent on activation of voltage-gated Ca^{2+} currents.[11] Lorazepam, however, may attenuate the amplitude of the $[Ca^{2+}]_i$ oscillations either by inhibiting influx of extracellular Ca^{2+} *via* capacitance-gated Ca^{2+} channels that open when intracellular Ca^{2+} stores are depleted or by reducing the amount of Ca^{2+} released from intracellular stores. The inhibitory effect of diazepam on the frequency of the $[Ca^{2+}]_i$ oscillations is likely to be more complex and may involve alterations in the binding of phenylephrine to α-adrenoreceptors *via* competitive, noncompetitive, or allosteric interactions. Alternatively, diazepam may alter more distal points in the signal transduction pathway for phenylephrine-induced $[Ca^{2+}]_i$ oscillations, such as the coupling between α-adrenoreceptor activation and the rate of inositol trisphosphate production[27] or binding to its receptor. It is also possible that some actions could be due to nonspecific effects of the benzodiazepine on the cell surface membrane. Additional experiments are required to address these possibilities.

Clinically Relevant Concentrations of Benzodiazepines in the Pulmonary Circulation

The clinically relevant free serum concentrations of benzodiazepines have been estimated at ≈ 1 μM for diazepam, midazolam, and lorazepam.[28-31] Clinically relevant concentrations of anesthetic agents *in vivo,* however, are difficult to ascertain and can vary over time depending on the speed of injection, volume of distribution, plasma protein concentration, and pH. All of the benzodiazepines used in this study avidly bind to serum proteins ($>90\%$), which significantly reduces the free level of drug in the serum capable of interacting with the tissue. Protein binding *in vivo,* however, is unlikely to be instantaneous, so free drug concentrations with a bolus induction dose are likely higher than those measured at equilibrium. In addition, first-pass concentrations in the pulmonary circulation are much higher than steady-state levels in the systemic circulation,[9] and the microkinetic behavior within the vascular space (drug transfer rate from serum to protein-bound state and cellular constituents) has not been defined. Despite the difficulty and uncertainty in estimating the *in vivo* concentrations and the likelihood that these estimates are different in different pathologic conditions (*e.g.,* hemodilution, liver disease, hypoproteinemia, hypovolemia), it seems unlikely that the concentrations of lorazepam and diazepam that had significant effects on $[Ca^{2+}]_i$ os-

cillations would be routinely encountered in clinical practice.

Summary

Lorazepam and diazepam but not midazolam have differential effects on phenylephrine-induced $[Ca^{2+}]_i$ oscillations in PASMCs. The precise cellular mechanisms that mediate these effects of benzodiazepines on $[Ca^{2+}]_i$ signaling in PASMCs remain to be elucidated.

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