Effects of L-type Ca^{2+} channel modulation on direct myocardial effects of diazepam and midazolam in adult rat ventricular myocytes

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Abstract

Purpose. Our objective was to determine whether an L-type Ca^{2+} channel modulation could alter myocardial depression induced by midazolam or diazepam in adult rat ventricular myocytes.

Methods. Freshly isolated rat ventricular myocytes were loaded with fura-2/AM and field-stimulated (0.3 Hz) at 28°C. Amplitude and timing of intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) and myocyte shortening were simultaneously monitored in individual cells.

Results. Midazolam (3–100 µM) caused a decrease in both peak [Ca^{2+}]$_i$ and shortening. Diazepam (30, 100 µM) increased myocyte shortening and peak [Ca^{2+}]$_i$; however, higher concentration of diazepam (300 µM) decreased shortening and peak [Ca^{2+}]$_i$. Bay K 8644 (0.01–10 µM), an L-type Ca^{2+} channel agonist, caused dose-dependent increases in peak [Ca^{2+}]$_i$ and shortening. In contrast, verapamil (0.1–50 µM), an L-type Ca^{2+} channel antagonist, caused dose-dependent decreases in peak [Ca^{2+}]$_i$ and shortening. Dose–response curves to benzodiazepines on peak [Ca^{2+}]$_i$ and shortening were not affected by pretreatment with Bay K 8644 (0.1 µM) or verapamil (1 µM). Diazepam (30, 100 µM), but not midazolam (3–30 µM), increased shortening and [Ca^{2+}]$_i$ in the presence or absence of L-type Ca^{2+} channel modulators. Diazepam (30 µM) and midazolam (10 µM) had no effect on peak [Ca^{2+}]$_i$ of a caffeine-induced [Ca^{2+}]$_i$ transient, which was used as a measure of SR Ca^{2+} content.

Conclusion. Midazolam and diazepam have differential effects on cardiac E-C coupling. Diazepam, but not midazolam, enhances cardiac E-C coupling independent of L-type Ca^{2+} channel modulation.

Key words Heart · Inotropes · Benzodiazepines · L-type Ca^{2+} channel · Cellular mechanisms

Introduction

Although benzodiazepines are characterized by relatively minor alterations in hemodynamic variables, they sometimes cause a profound decrease in blood pressure [1–3]. In vitro experiments, these agents have a direct myocardial depressant effect [4–9]. However, the mechanisms underlying the myocardial depressant effects of these benzodiazepines are not fully understood.

In cardiac myocytes, Ca^{2+} influx across the sarcolemma via L-type Ca^{2+} channels is generally accepted as the trigger of sarcoplasmic reticulum (SR) Ca^{2+} release [10,11]. Therefore, inhibition of the L-type Ca^{2+} channels is one possible mechanism for anesthetic-induced myocardial depression. Midazolam has been reported to reduce L-type Ca^{2+} channel current (I$_{Ca}$) in a dose-dependent manner in isolated canine ventricular myocytes [12]. In cultured neonatal rat ventricular myocytes, an L-type Ca^{2+} channel agonist, Bay K 8644, attenuated the myocardial depression caused by diazepam and midazolam [8]. This direct and indirect evidence indicates that inhibition of I$_{Ca}$ could play an important role in direct myocardial depression caused by diazepam and midazolam. However, several issues should be discussed to understand the direct myocardial depressant effect of the benzodiazepines. First, evidence has been accumulated that SR Ca^{2+} release may be regulated independently from I$_{Ca}$ in some situations [11,13]. If this is the case, benzodiazepines may alter cardiac excitation-contraction (E-C) coupling via a different effect on I$_{Ca}$ and SR Ca^{2+} release. Second, the immature myocardium is known to have different characteristics compared with adult (mature) myocardium [14]. Therefore, it is important to know the direct effects of the benzodiazepines on E-C coupling and Ca^{2+} signaling at the cellular level in adult rat ventricular myocytes.

The purpose of the present study was to determine whether an L-type Ca^{2+} channel modulation could alter

the myocardial depression induced by midazolam or diazepam in adult rat ventricular myocytes. This experimental model allowed us to simultaneously measure changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and myocyte shortening independent of any neural, humoral, or locally derived factors.

Methods and materials

This study was approved by The Cleveland Clinic Foundation's Animal Care and Use Committee. Isolated adult ventricular myocytes from rat hearts were obtained as previously described [15,16]. In brief, the hearts were excised, cannulated via the aorta, attached to a modified Langendorff perfusion apparatus, and perfused with oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit buffer (KHB, 37°C) containing the following (in mM): 118 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 1.2 $CaCl_2$, 37.5 $NaHCO_3$, 16.5 dextrose, pH 7.35. Following a 5-min equilibration period, the perfusion buffer was changed to Ca^{2+}-free KHB containing 30 mg collagenase type II (Worthington Biochemical, Freehold, NJ, USA; lot M6C152, 347 U/ml). Following collagenase digestion (20 min), the ventricles were minced and shaken in KHB and the resulting cellular digest washed, filtered, and resuspended in phosphate-free 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES)-buffered saline (HBS) containing the following (in mM): 118 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 11 dextrose, 25 HEPES, 5 pyruvate, pH 7.35, and vigorously bubbled immediately before use with 100% O_2. Typically, 6–8 × 10^6 cells per rat heart were obtained using this procedure. Viability, as assessed by the percentage of cells retaining a rod-shaped morphology with no blebs or granulations, was routinely between 80% and 90%. Myocytes were suspended in HBS (1 × 10^6 cells/ml) and stored in an O_2 hood until used.

Simultaneous measurement of myocyte shortening and $[Ca^{2+}]_i$ was performed as previously described [15,16]. Ventricular myocytes (0.5 × 10^6 cells/ml) were incubated in HBS containing 2 µM fura-2/AM at 37°C for 20 min. Fura-2-loaded ventricular myocytes were placed in a temperature-regulated (28°C) chamber (Bioptechs, Butler, PA, USA) mounted on the stage of an Olympus IX-70 (Olympus America, Lake Success, NY, USA) inverted fluorescence microscope. The chamber volume was 1.5 ml. The cells were superfused continuously with HBS at a flow rate of 2 ml/min and field stimulated via bipolar platinum electrodes at a frequency of 0.3 Hz and a duration of 5 ms using a Grass SD9 stimulator (Grass Instruments, West Warwick, RI, USA). Myocytes were chosen for study according to the following criteria: (1) rod-shaped appearance with clear striations and no membrane blebs;

(2) a negative staircase of twitch performance on stimulation from rest; and (3) the absence of spontaneous contractions.

Fluorescence measurements were performed on individual ventricular myocytes using a dual-wavelength spectrofluorometer (Deltascan RFK6002; Photon Technology International, South Brunswick, NJ, USA) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The cells were also illuminated with red light at a wavelength above 600 nm for simultaneous video edge detection. An additional postspecimen dichroic mirror deflected light at wavelengths greater than 600 nm into a charge-coupled device (CCD) video camera (Phillips VC 62505T; Marshall Electronics, Culver City, CA, USA) for measurement of myocyte shortening and relengthening. The fluorescence sampling frequency was 100 Hz, and data were collected using a software package from Photon Technology International (Felix). $[Ca^{2+}]_i$ was estimated by comparing the cellular fluorescence ratio with fluorescence ratios acquired using fura-2 (free acid) in buffers containing known Ca^{2+} concentrations.

Simultaneous measurement of cell shortening was monitored using a video edge detector (Crescent Electronics, Sandy, UT, USA) with 16-ms temporal resolution. The video edge detector was calibrated using a stage micrometer so that cell lengths during shortening and relengthening could be monitored. Lab View (National Instruments, Austin, TX, USA) was used for data acquisition of cell shortening using a sampling rate of 100 Hz.

Fluorescence Data for $[Ca^{2+}]_i$ were imported into Labview, where both the $[Ca^{2+}]_i$ and myocyte contractile responses were analyzed synchronously and simultaneously. The following parameters were calculated for each individual contraction: diastolic $[Ca^{2+}]_i$ and cell length; systolic $[Ca^{2+}]_i$ and cell length; and change in $[Ca^{2+}]_i$ and twitch amplitude. Parameters from 15 contractions ($[Ca^{2+}]_i$ and shortening) were averaged to obtain mean values at baseline and in response to the various interventions. Averaging the parameters over time minimized beat-to-beat variation.

Myocyte length in response to field stimulation was measured (µm) and is expressed as the change from resting cell length (twitch amplitude). Changes in twitch amplitude in response to the interventions are expressed as a percent of baseline shortening. Changes in the timing parameters were measured in milliseconds (ms) and were normalized to changes in amplitude. The rising phase of $[Ca^{2+}]_i$ and shortening (typical value, approximately 150 ms) was measured with enough time resolution (10 ms) to accurately compute the timing parameters. Changes in $[Ca^{2+}]_i$ were measured as the change in the 340/380 ratio from baseline. Changes in the 340/380 ratio in response to the interventions were

expressed as a percent of the control response in the absence of any intervention.

Experimental protocols

Protocols were designed such that each cell could be used as its own control.

Protocol 1: Effects of L-type Ca^{2+} channel modulators on $[Ca^{2+}]_i$ and myocyte shortening. Changes in myocyte shortening and $[Ca^{2+}]_i$ during exposure to Bay K 8644, an L-type Ca^{2+} channel agonist, or to verapamil, an L-type Ca^{2+} channel antagonist, were determined. Baseline measurements were collected from individual myocytes for 1.5 min in the absence of any intervention. Myocytes were exposed to four concentrations of each drug (Bay K 8644: 0.01, 0.1, 1, 10 μM; verapamil: 0.1, 1, 10, 50 μM), achieved by exchanging the buffer in the dish with new buffer containing each drug at the desired concentration. Data were acquired for 1.5 min following a 5-min equilibration period in the presence of the drug. Individual myocytes were exposed to only one drug. Nifedipine, an L-type Ca^{2+} channel antagonist, could not be used because of its light-sensitive property.

Protocol 2: Effects of L-type Ca^{2+} channel modulators on dose-dependent effects of midazolam and diazepam. To determine whether midazolam or diazepams alter Ca^{2+} entry through L-type Ca^{2+} channels, we examined the effect of midazolam and diazepam on $[Ca^{2+}]$ and shortening in the presence or absence of Bay K8644, an L-type Ca^{2+} channel agonist, or verapamil, an L-type Ca^{2+} channel antagonist. Dose–response curves to each benzodiazepine on shortening and $[Ca^{2+}]_i$ were generated in the presence or absence of the EC_{50} dose for each modulator (Bay K 8644: 0.1 μM; verapamil: 1 μM). The myocytes served as their own controls: baseline data were collected at 3 min after Bay K 8644 or verapamil, before benzodiazepine administration. To estimate the effect of the benzodiazepines under maximal activation of the L-type Ca^{2+} channels, we examined the effect of midazolam and diazepam on $[Ca^{2+}]$ and shortening in the presence or absence of Bay K8644 (10 μM).

Protocol 3: Effect of midazolam and diazepam on SR Ca^{2+} stores. To determine whether midazolam or diazepam alter Ca^{2+} release from intracellular Ca^{2+} stores, we measured caffeine-induced $[Ca^{2+}]_i$ release in the presence or absence of the benzodiazepines. Baseline $[Ca^{2+}]_i$ transients were collected from individual myocytes for 1.5 min. Midazolam (10 μM), diazepam (30 μM), or ryanodine (10 μM, which activates the Ca^{2+}-release channel and depletes intracellular Ca^{2+} stores), were then added to the superfusion buffer and allowed to equilibrate for 5 min. Field stimulation of the myocyte was discontinued, and caffeine (20 mM) was applied to the cell 15 s later. The amplitude of the $[Ca^{2+}]_i$ transient induced by caffeine was compared to peak $[Ca^{2+}]_i$ in response to steady-state stimulation before addition of the respective drugs and is reported as a percent of the control amplitude.

Materials

Midazolam and diazepam were obtained from the Cleveland Clinic Pharmacy. Caffeine, Bay K8644, and verapamil were purchased from Sigma Chemical (St. Louis, MO, USA). Ryanodine was obtained from Research Biochemicals (Natick, MA, USA).

Statistical analysis and data presentation

Each experimental protocol was performed on multiple myocytes from the same heart and repeated in at least four hearts. Results obtained from myocytes in each heart were averaged so that all hearts were weighted equally. The dose-dependent effects of midazolam or diazepam on myocyte shortening and $[Ca^{2+}]_i$ were assessed using one-way analysis of variance (ANOVA) with repeated measures and the Bonferonni–Dunn post hoc test. Comparisons between groups were made by two-way ANOVA. Results are expressed as means ± SEM. Differences were considered statistically significant at $P < 0.05$.

Results

Effect of Bay K 8644 on myocyte shortening and $[Ca^{2+}]_i$

Figure 1A demonstrates that addition of Bay K 8644, an L-type Ca^{2+} channel agonist, to an individual, field-stimulated ventricular myocyte results in dose-dependent increase of myocyte shortening and a concomitant increase in peak $[Ca^{2+}]_i$. The positive inotropic effect of Bay K 8644 was completely reversed after washout. Bay K 8644 had no effect on resting $[Ca^{2+}]_i$ or cell length. The summarized data for myocyte shortening and $[Ca^{2+}]_i$ are shown in Fig. 1B,C. Bay K 8644 (0.01–10 μM) caused a dose-dependent increase in myocyte shortening and $[Ca^{2+}]_i$.

Effect of verapamil on myocyte shortening and $[Ca^{2+}]_i$

Figure 2A demonstrates that addition of verapamil, an L-type Ca^{2+} channel antagonist, to an individual, field-stimulated ventricular myocyte results in dose-dependent decrease of myocyte shortening and a

A

B
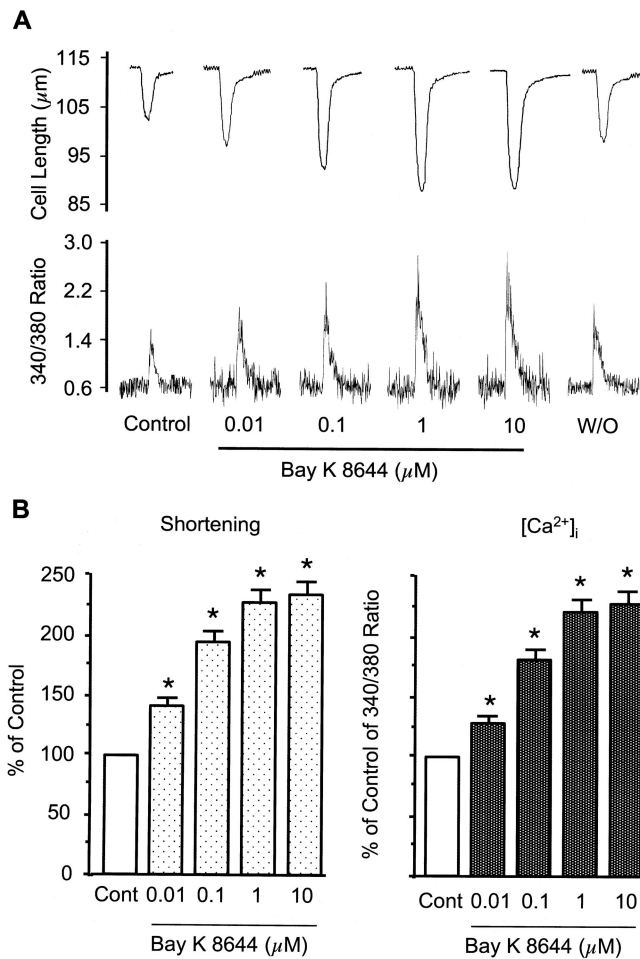
Shortening $[Ca^{2+}]_i$

Fig. 1. A Representative trace demonstrating the dose-dependent effects of Bay K 8644 on myocyte shortening (*top*) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (*bottom*) in a single ventricular myocyte. Changes in cell length were measured in micrometers (μm). $[Ca^{2+}]_i$ was measured as the 340/380 ratio. Bay K 8644 increased both myocyte shortening and $[Ca^{2+}]_i$ in a dose-dependent manner; the effects were reversed after washout (*W/O*). **B** Summarized data for the effect of Bay K 8644 on the amplitude of myocyte shortening and $[Ca^{2+}]_i$. Results are expressed as a percent of control (*Cont*) in the absence of any intervention. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 20$ cells/5 hearts

A

B

Shortening $[Ca^{2+}]_i$

Fig. 2. A Representative trace demonstrating the dose-dependent effects of verapamil on myocyte shortening (*top*) and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (*bottom*) in a single ventricular myocyte. Changes in cell length were measured in μm. $[Ca^{2+}]_i$ was measured as the 340/380 ratio. Verapamil decreased both myocyte shortening and $[Ca^{2+}]_i$ in a dose-dependent manner; the effects were reversed after washout (*W/O*). **B** Summarized data for the effect of verapamil on the amplitude of myocyte shortening and $[Ca^{2+}]_i$. Results are expressed as a percent of control (*Cont*) in the absence of any intervention. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 20$ cells/5 hearts

concomitant decrease in peak $[Ca^{2+}]_i$. The negative inotropic effect of verapamil was completely reversed after washout. Verapamil had no effect on resting $[Ca^{2+}]_i$ or cell length. The summarized data for myocyte shortening and $[Ca^{2+}]_i$ are shown in Fig 2B,C. Verapamil (0.1–50μM) caused a dose-dependent decrease in myocyte shortening and $[Ca^{2+}]_i$.

Effect of Bay K 8644 on myocardial depressant effect of midazolam or diazepam

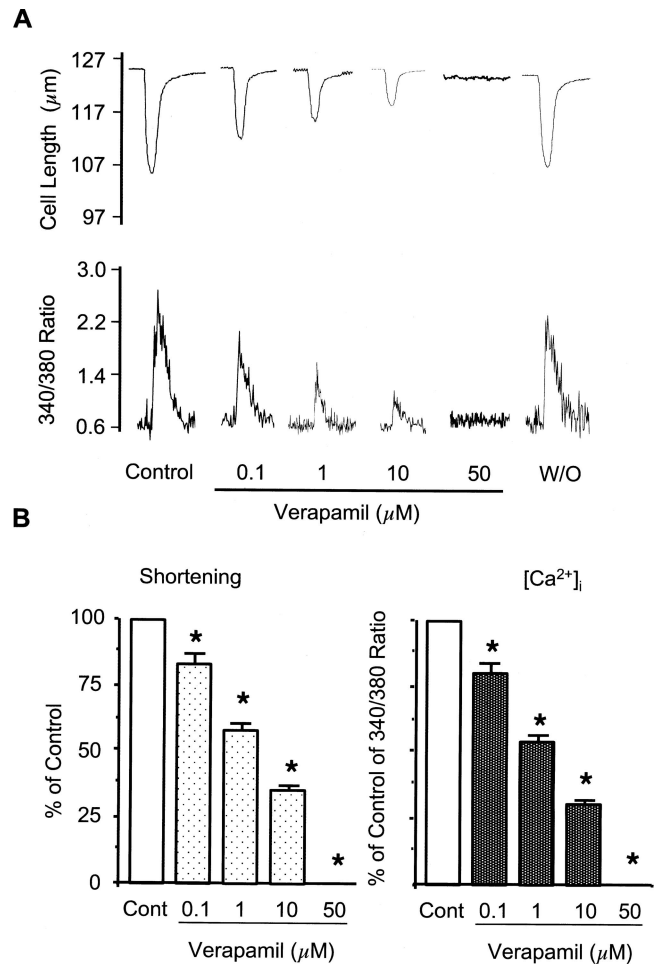Midazolam had no effect on $[Ca^{2+}]_i$ or shortening at concentrations up to 30μM, whereas 100μM midazolam

caused profound myocardial depression (Fig. 3). In the presence of Bay K8644 (0.1 μM), midazolam (10, 30μM) had no effect on $[Ca^{2+}]_i$ or shortening. Inhibition of $[Ca^{2+}]_i$ and shortening was observed with the highest concentration of midazolam (100μM). Midazolam (100μM) had no effect on $[Ca^{2+}]_i$ (98% ± 3% of control) or shortening (98% ± 3% of control) when the L-type Ca^{2+} channel was maximally activated with 10μM Bay K8644 (Fig. 4). Diazepam exerted a dose-dependent, biphasic effect on myocyte shortening and $[Ca^{2+}]_i$ (Fig. 5). Increases in myocyte shortening and peak $[Ca^{2+}]_i$ were observed with 30 and 100μM diazepam, whereas marked myocardial depression was observed at the
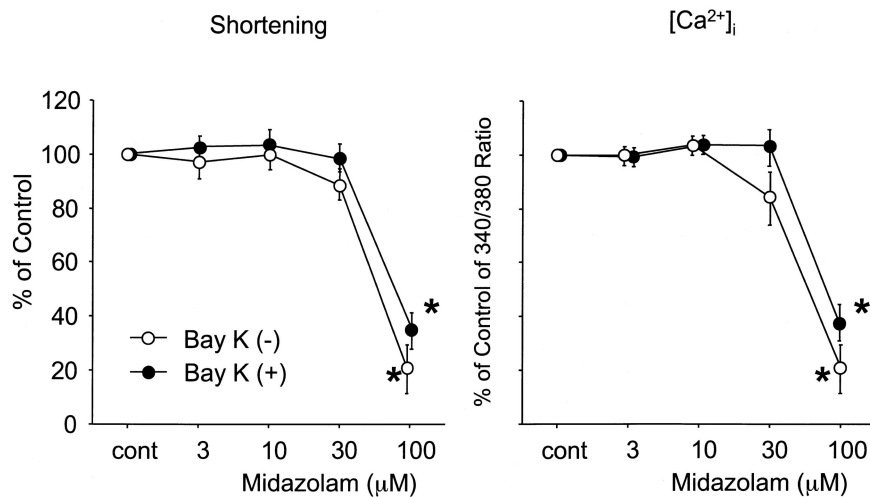
Fig. 3. Summarized data showing the effect of midazolam on myocyte shortening (*left*) and $[Ca^{2+}]_i$ (*right*) in the presence or absence of Bay K8644. Following pretreatment with the EC_{50} dose of Bay K8644 (0.1 μM), midazolam was added at the concentrations depicted in the figure. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts
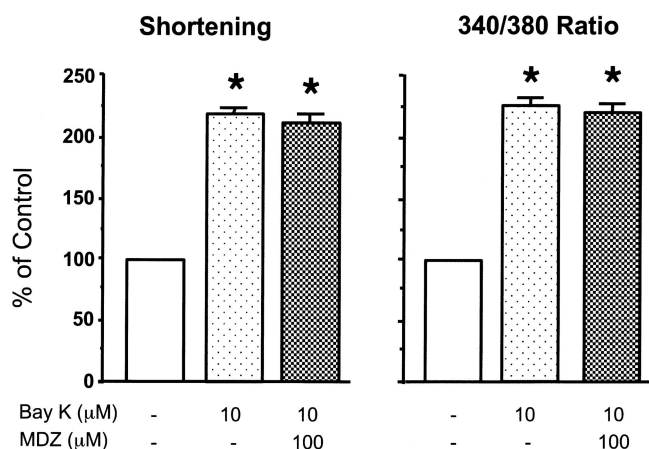

Fig. 4. Summarized data showing the effect of midazolam (*MDZ*) on myocyte shortening and $[Ca^{2+}]_i$ in the presence or absence of Bay K8644 (*BayK*). Following pretreatment with the highest dose of Bay K8644 (10 μM), midazolam (100 μM) was added. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts

highest concentration tested (300 μM). This myocardial depressant effect was readily reversible following washout of diazepam. The vehicle for diazepam, an alcohol:propylene glycol mixture, had no significant effect on myocyte shortening or peak $[Ca^{2+}]_i$. Diazepam had no effect on resting $[Ca^{2+}]_i$ or cell length. After treatment with 0.1 μM Bay K 8644, myocyte shortening and $[Ca^{2+}]_i$ increased by 170% ± 10% and 175% ± 10%, respectively. In the presence of Bay K 8644, diazepam (30, 100 μM) caused a further increase in $[Ca^{2+}]_i$ and shortening, but decreased $[Ca^{2+}]_i$ and shortening at the highest concentration tested (300 μM; see Fig. 5). However, the magnitude of increases in shortening and

$[Ca^{2+}]_i$ was identical in the presence or absence of Bay K 8644. Diazepam (300 μM) had no effect on $[Ca^{2+}]_i$ (97% ± 4% of control) or shortening (98% ± 3% of control) when the L-type Ca^{2+} channel was maximally activated with 10 μM Bay K8644 (Figure 6).

Effect of verapamil on myocardial depressant effect of midazolam or diazepam

After treatment with 1 μM verapamil, myocyte shortening and $[Ca^{2+}]_i$ decreased by 55% ± 3% and 62% ± 3%, respectively. Verapamil had no effect on a dose-dependent decrease in $[Ca^{2+}]_i$ or shortening at every midazolam concentration tested (Fig. 7). In the presence of verapamil, midazolam (30 μM) had no effect on $[Ca^{2+}]_i$ (99% ± 3% of control) or shortening (99% ± 2% of control) but nearly abolished both $[Ca^{2+}]_i$ and shortening at the highest concentration tested (100 μM). Verapamil also had no significant effect on diazepam-induced biphasic effect on myocyte shortening and $[Ca^{2+}]_i$ (Fig. 8). Diazepam (30, 100 μM) increased $[Ca^{2+}]_i$ and shortening in the presence of verapamil (1 μM) but almost abolished both $[Ca^{2+}]_i$ and shortening at the highest concentration tested (300 μM; Fig. 8).

Effect of midazolam and diazepam on SR Ca^{2+} stores

To measure SR Ca^{2+} content, we applied caffeine (20 mM) after the 15-s rest period. Figure 9 shows that neither midazolam 10 μM nor diazepam 30 μM altered the amplitude of the caffeine-releasable pool of Ca^{2+} compared with that observed the control response to caffeine. In contrast, ryanodine (10 μM), which activates the Ca^{2+} release channel and depletes intracellular stores, abolished the caffeine-induced increase in $[Ca^{2+}]_i$ (Fig. 9).
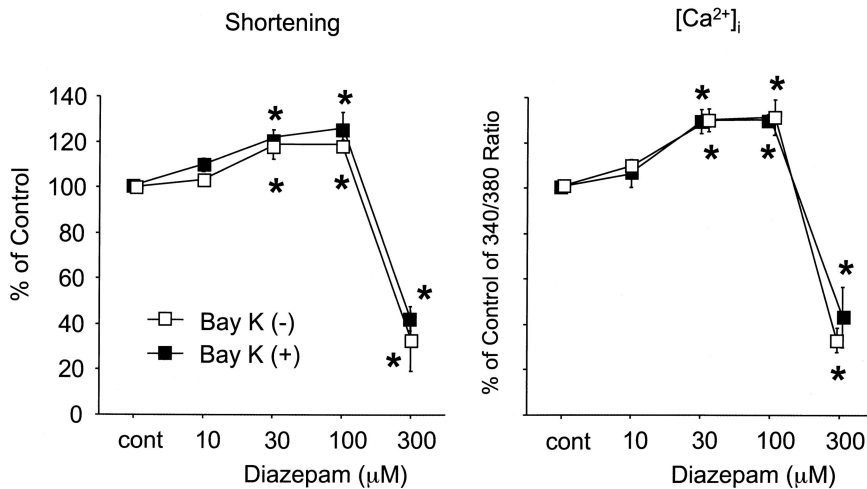
Shortening

[Ca²⁺]ᵢ

Fig. 5. Summarized data showing the effect of diazepam on myocyte shortening (*left*) and $[Ca^{2+}]_i$ (*right*) in the presence or absence of Bay K8644. Following pretreatment with the EC_{50} dose of Bay K8644 ($0.1\,\mu M$), diazepam was added at the concentrations depicted in the figure. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts
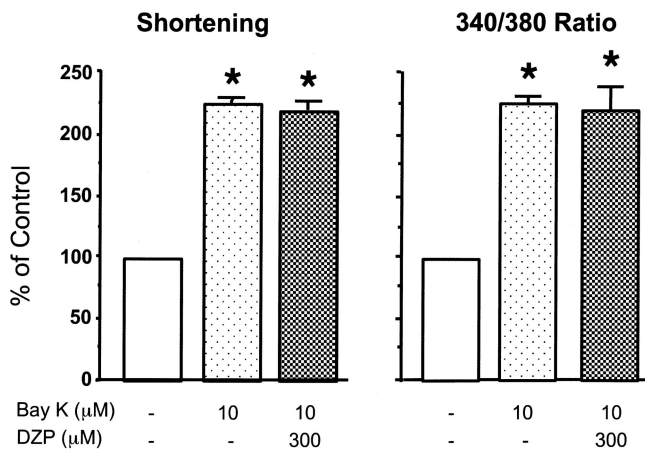
Shortening   340/380 Ratio

Fig. 6. Summarized data showing the effect of diazepam (*DZP*) on myocyte shortening (*left*) and $[Ca^{2+}]_i$ (*right*) in the presence or absence of Bay K8644 (*BayK*). Following pretreatment with the highest dose of Bay K8644 ($10\,\mu M$), midazolam ($100\,\mu M$) was added. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts

Discussion

In this study, we investigated the effects of L-type Ca^{2+} channel modulators on the myocardial depressant effects of midazolam and diazepam in individual, field-stimulated adult rat ventricular myocytes. We found that midazolam decreased shortening and peak $[Ca^{2+}]_i$ in response to field stimulation at the highest concentration tested. In contrast, diazepam elicited a biphasic inotropic response with a modest increase in shortening and peak $[Ca^{2+}]_i$ at lower concentrations and caused a potent myocardial depressant effect and marked decrease in peak $[Ca^{2+}]_i$ at the highest concentration tested. Bay K 8644 ($0.01–10\,\mu M$), an L-type Ca^{2+} channel agonist, increased myocyte shortening and $[Ca^{2+}]_i$ in

a dose-dependent manner. In contrast, verapamil ($0.1–50\,\mu M$), an L-type Ca^{2+} channel antagonist, decreased myocyte shortening and $[Ca^{2+}]_i$ in a dose-dependent manner. However, the dose–response curves to the two benzodiazepines for shortening and $[Ca^{2+}]_i$ were not affected in the presence of Bay K 8644 ($0.1\,\mu M$) or verapamil ($1\,\mu M$). Neither benzodiazepine altered the amount of Ca^{2+} released from the SR in response to caffeine at the lower concentrations tested.

The hemodynamic effects of midazolam and diazepam have been studied extensively in vivo [1–3,17]. Several studies have also described the direct negative inotropic effects of benzodiazepines in vitro [4–8]. These observations are consistent with the reduction in myocyte shortening by midazolam and diazepam observed in this study.

Using the L-type Ca^{2+} channel modulators Bay K8644 (agonist) and verapamil (antagonist), we examined whether the effects of midazolam and diazepam on myocyte shortening and $[Ca^{2+}]_i$ could be explained by changes in L-type Ca^{2+} channel activity. In the presence or absence of Bay K8644 (EC_{50}), midazolam only had an inhibitory effect at the highest concentration tested ($100\,\mu M$). Moderate activation of the sarcolemmal L-type Ca^{2+} channel had no effect on myocardial depressant effect of midazolam (see Fig. 3). However, maximal activation of the L-type Ca^{2+} channel completely abolished the myocardial depression of midazolam at the highest concentration tested. These data suggest that the negative inotropic effect of midazolam is mediated, at least in part, via a reduction in L-type Ca^{2+} channel activity. Our results with verapamil also support this concept. These data are also consistent with out previous report that inhibition of the L-type Ca^{2+} channel is important in the direct myocardial depressant effects of midazolam and diazepam in cultured ventricular myocytes [8]. Moreover, Buljubasic et al. reported that $60\,\mu M$ midazolam decreased the
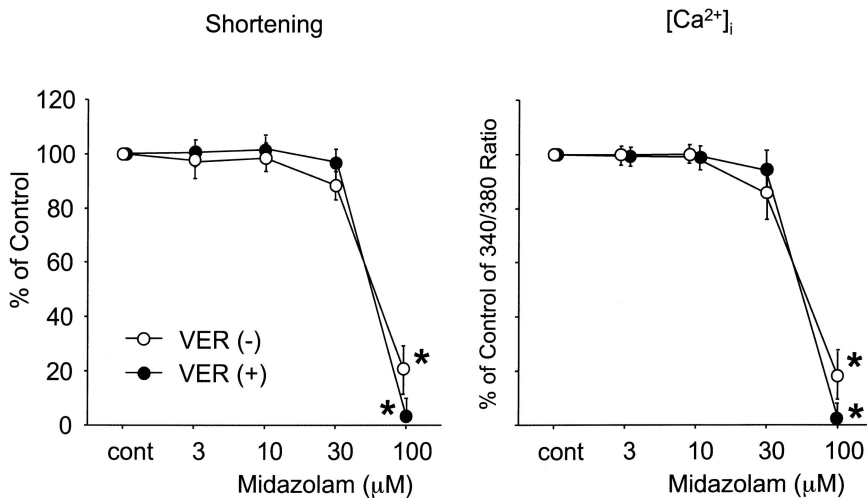
Shortening

[Ca²⁺]ᵢ

Fig. 7. Summarized data showing the effect of midazolam on myocyte shortening (*left*) and $[Ca^{2+}]_i$ (*right*) in the presence or absence of verapamil (*VER*). Following pretreatment with the EC_{50} dose of verapamil (1 μM), midazolam was added at the concentrations depicted in the figure. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts
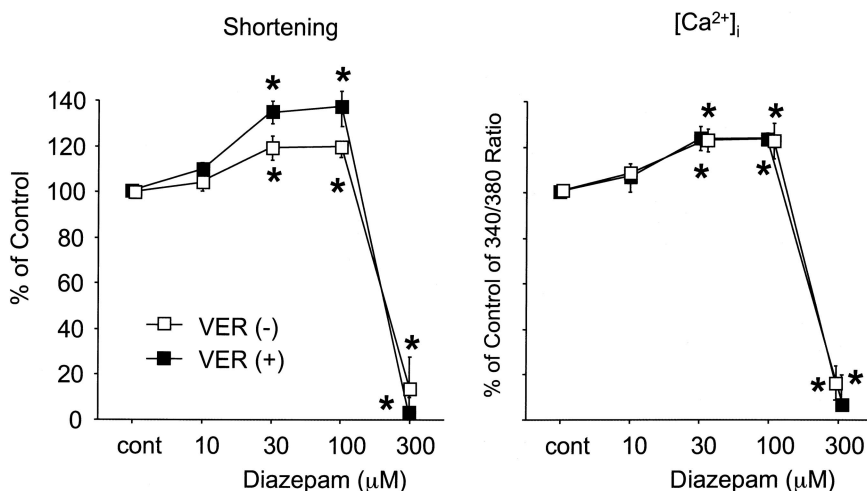
Shortening

[Ca²⁺]ᵢ

Fig. 8. Summarized data showing the effect of diazepam on myocyte shortening (*left*) and $[Ca^{2+}]_i$ (*right*) in the presence or absence of verapamil (*VER*). Following pretreatment with the EC_{50} dose of verapamil (1 μM), diazepam was added at the concentrations depicted in the figure. *Asterisks* indicate significant change from control ($P < 0.05$); $n = 10$ cells/5 hearts
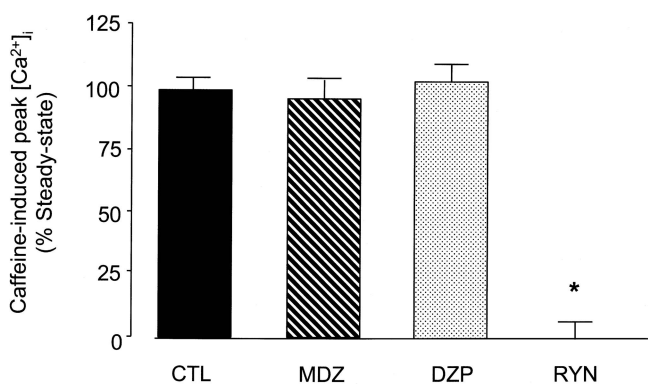
Fig. 9. Summarized data for the effects of midazolam (*MDZ*; 10 μM), diazepam (*DZP*; 30 μM), and ryanodine (*RYN*; 10 μM) on caffeine-induced $[Ca^{2+}]_i$ release. The amplitude of the caffeine-induced $[Ca^{2+}]_i$ transient was compared to the amplitude of the field-stimulated $[Ca^{2+}]_i$ transient. Results are expressed as a percent of the field-stimulated steady-state amplitude. *Asterisk* indicates significant change from control (*CTL*) ($P < 0.05$); $n = 15$ cells/6 hearts for control group; $n = 8$ cells/4 hearts for other groups

peak Ca^{2+} current (I_{Ca}) by 47% in canine myocardial cells using the whole-cell voltage-clamp technique [12].

In our present study, we observed a small positive inotropic effect with low concentrations of diazepam. At least three mechanisms could be involved in this effect. First, this effect is likely caused by an increase in Ca^{2+} influx across the sarcolemma, because a concomitant increase in peak $[Ca^{2+}]_i$ was observed without any increase in the size of the caffeine-releasable pool (SR Ca^{2+} store). However, it is unlikely that diazepam acts as the L-type Ca^{2+} channel agonist because it reduces I_{Ca} in a dose-dependent manner [18]. Second, sensitization of the Ca induced Ca release (CICR) mechanism of the SR resulting in the release of a larger amount of Ca^{2+} from the SR could be involved. This effect has been reported for some inhalational anesthetics [19], Ca-calmodulin-dependent protein kinase (CaMKII), and the immunosupressant drug FK506 [11]. Third, another possible mechanism for positive inotropic effect of diazepam is blockade of the outward K^+ channel current.

Blockade of the outward K⁺ channel can affect the duration of the action potential and the rate of repolarization. Although results from patch-clamp studies support this idea, the concentrations of diazepam for inhibiting the outward K^+ channel current were much higher than those for inhibiting the Ca^{2+} channel current [12,18]. Therefore, the positive inotropic effect may have been masked in multicellular preparations or in vivo by the benzodiazepines acting at multiple sites (central and local), thereby counteracting the direct cardiac effects.

The results of this study need to be interpreted in the context of the experimental conditions (low temperature, 28°C, and low frequency of stimulation, 0.3Hz). These experimental conditions are necessary to maintain myocyte viability throughout the time course of the experiments. Peak plasma concentrations during induction are approximately 0.5μM for midazolam (96% protein binding) [1] and 0.7μM (96% protein binding) for diazepam [20]. All induction agents are known to bind to plasma protein, which decreases their effective circulating concentration. As a result, the actual free plasma concentration available for binding to tissues is a fraction of the total plasma concentration. Thus, it appears unlikely that the cardiovascular effects of clinically srelevant doses of midazolam and diazepam in vivo are mediated by their direct action on cardiac contractility. However, the microkinetic behavior within the vascular space has not been defined, and small changes in the amount or binding capacity of proteins could result in significant increases in the free plasma concentration of the benzodiazepines. Not only is there uncertainty in calculating the in vivo concentration of benzodiazepines under normal circumstances, but the free plasma concentration would certainly be higher when serum protein concentration is reduced (e.g., hemodilution, liver disease, hypoproteinemia). Similar findings regarding the inhibitory potency of benzodiazepines on $[Ca^{2+}]_i$ signaling in pulmonary vascular smooth muscle have been reported by our laboratory [21].

Summary

The negative inotropic effects of midazolam appear to be mediated entirely by a decrease in peak $[Ca^{2+}]_i$, which may involve a decrease in sarcolemmal Ca^{2+} influx. Diazepam caused a positive inotropic effect at low doses, whereas a potent negative inotropic effect was observed at the highest concentration tested. The positive inotropic effect is likely mediated via an increase in sarcolemmal Ca^{2+} influx or sensitization of CICR, rather than increased Ca^{2+} content within SR stores. The cardiodepressant effect of diazepam appears to be mediated by a decrease in L-type Ca^{2+} channel activity that results in a decrease in the availability of $[Ca^{2+}]_i$.

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