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Inhibitory Effects of Diazepam and Midazolam on Ca^{2+} and K^+ Channels in Canine Tracheal Smooth Muscle Cells

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Background: **Benzodiazepines have a direct bronchodilator action in airway smooth muscle, but the mechanisms by which these agents produce muscle relaxation are not fully understood. The current study was performed to identify the effects of the benzodiazepines diazepam and midazolam on Ca^{2+} and K^+ channels in canine tracheal smooth muscle cells.**

Methods: **Whole-cell patch-clamp recording techniques were used to evaluate the effects of the benzodiazepines diazepam (10^{-8} to 10^{-3} M) and midazolam (10^{-8} to 10^{-3} M) on inward Ca^{2+} and outward K^+ channel currents in dispersed canine tracheal smooth muscle cells. The effects of the antagonists flumazenil (10^{-5} M) and PK11195 (10^{-5} M) on these channels were also studied.**

Results: **Each benzodiazepine tested significantly inhibited Ca^{2+} currents in a dose-dependent manner, with 10^{-6} M diazepam and 10^{-5} M midazolam each causing approximately 50% depression of peak voltage-dependent Ca^{2+} currents. Both benzodiazepines promoted the inactivated state of the channel at more-negative potentials. The Ca^{2+}-activated and voltage-dependent K^+ currents were inhibited by diazepam and midazolam ($> 10^{-5}$ M and $> 10^{-4}$ M, respectively). Flumazenil and PK11195 had no effect on these channel currents or on the inhibitory effects of the benzodiazepines.**

Conclusions: **Diazepam and midazolam had inhibitory effects on voltage-dependent Ca^{2+} channels, which lead to muscle relaxation. However, high concentrations of these agents were necessary to inhibit the K^+ channels. The lack of antagonized effects of their antagonists is related to the non-γ-aminobutyric acid–mediated electrophysiologic effects of benzodiazepines on airway smooth muscle contractility. (Key words: Ca^{2+}-activated K^+ channel; flumazenil; PK11195; voltage-dependent delayed rectifier K^+ channel.)**

BENZODIAZEPINES, especially midazolam, have been used widely for sedation and to induce general anesthesia. In addition to their hypnotic action, these agents have a direct relaxing effect on airway smooth muscle[1,2] and vascular smooth muscle.[3,4] Because the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) plays a central role in the regulation of airway smooth muscle tone,[5,6] a possible mechanism for the relaxation produced by benzodiazepines is a decrease in $[Ca^{2+}]_i$. Yoshimura *et al.*[7] used the Ca^{2+} indicator fura-2 to show that relaxation of contracted porcine tracheal smooth muscle by midazolam at clinically relevant concentrations was associated with a decrease in $[Ca^{2+}]_i$. Sustained contraction of airway smooth muscle requires the continued entry of extracellular Ca^{2+},[8] and the blockade of voltage-dependent Ca^{2+} channels (VDCCs) suppresses the sustained increase in $[Ca^{2+}]_i$ in agonist-stimulated tracheal smooth muscle.[9] We hypothesized, therefore, that benzodiazepines reduce $[Ca^{2+}]_i$ by inhibiting VDCC.

Conversely, the open-state probability of VDCC depends on the plasma membrane potential, which is regulated by K^+-selective channels.[10,11] One other potential mechanism for bronchodilation by benzodiazepines is enhanced K^+ conductance, leading to a decrease in VDCC opening and thus to muscle relaxation.[12]§

In the current study, we used whole-cell patch-clamp techniques to identify the direct effects of the benzodiazepines diazepam and midazolam on Ca^{2+} and K^+ channels in freshly dispersed canine tracheal smooth muscle cells. We also evaluated the antagonized effects of the benzodiazepine antagonists flumazenil and PK11195

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§ Ducros L, Plaisance P, Joseph T, Bard M, Salmeron S, Payen D, Lecarpentier Y: Determinants of ketamine-induce bronchodilation in guinea pig tracheal smooth muscle (abstract). Am J Respir Crit Care Med 1996; 153:A741

(specific central-type and specific peripheral-type antagonists, respectively) on the Ca^{2+} and K^+ channels.

Materials and Methods

Preparation of Dispersed Canine Tracheal Smooth Muscle Cells

The Sapporo Medical University Ethical Committee on Animal Research approved the study. Adult mongrel dogs weighing 9–12 kg were anesthetized with 10 mg/kg intramuscular ketamine and killed by exsanguination. The tracheas were excised quickly and placed in modified Krebs' solution equilibrated with 95% oxygen and carbon dioxide at 4°C (composed of 118 mM NaCl, 4.7 mM KCl, 21 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, and 2.5 mM $CaCl_2$; *p*H ~7.4). Cells were dispersed according to previously described methods.[13,14] Briefly, tracheal smooth muscle was minced and incubated for 10 min in Ca^{2+}-free modified Tyrode's solution at room temperature (22–24°C). The modified Tyrode's solution contained 135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 5 mM N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), and 0.1% (wt/vol) bovine serum albumin, with the *p*H adjusted to 7.4 with 0.5 M tris-[hydroxymethy]aminomethane (Tris). The tissue was digested for 20 min at 37°C in Ca^{2+}-free modified Tyrode's solution that contained 0.08% (wt/vol) collagenase, 0.05% trypsin inhibitor, and 0.03% protease. Cells were dispersed by trituration, filtered through nylon mesh, and centrifuged. The pellet was resuspended in a modified Kraftbrühe solution[15] and stored at 4°C for as long as 5 h before being used. The modified Kraftbrühe solution contained 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 5 mM Na_2ATP, 5 mM pyruvic acid, 5 mM creatine, 20 mM taurine, 5 mM β-hydroxybutyrate, and 0.1% (wt/vol) fatty acid-free bovine serum albumin, with the *p*H adjusted to 7.25 with Tris.

Whole-cell Patch-clamp Recording

All experiments were performed at room temperature (22–24°C). Micropipettes were pulled from soda lime hematocrit tubing (GC-1.5; Narishige, Tokyo, Japan) using a two-stage puller (model PP-83, Narishige) and were heat polished. These had resistances of 3 to 5 MΩ when filled with solution. An aliquot (approximately 0.5 ml) of the cell suspension was placed in a perfusion chamber on the stage of an inverted microscope (IX-70; Olympus, Tokyo, Japan). At ×600 magnification, a three-dimensional oil-driven micromanipulator (ONM-1; Narishige) was used to position the patch pipettes against the membrane of the tracheal smooth muscle cells. After obtaining a high-resistance seal (5–50 GΩ) with slight suction (5–20 cm water), the patch membrane was disrupted by strong negative pressure, which allowed the voltage of the entire cell membrane to be controlled[16] and permitted the pipette solution to diffuse into the cytoplasm. Membrane currents were monitored using a CEZ-2400 patch-clamp amplifier (Nihon Kohden, Tokyo, Japan), and the amplifier output was low-pass filtered at 2,000 Hz. Leak currents, estimated by appropriate scaling of currents during 20-mV hyperpolarizing pulses, were subtracted from each of these records. Membrane capacitance and series resistance were compensated for by using the internal circuitry of the patch-clamp amplifier. All data were digitized (10,000 samples per s), stored on a hard disk, and analyzed using a 8100/100AV Power Macintosh computer (Apple, Cupertino, CA) using the Pulse+PulseFit 8.02 and Igor Pro 2.04 analysis software programs (Heka, Wiesenstrasse, Lambrecht, Germany).

To measure inward Ca^{2+} currents (I_{Ca}) through VDCCs, recording solutions were chosen to inhibit K^+ currents and enhance Ca^{2+} currents. The pipette solution contained 130 mM CsCl, 4 mM $MgCl_2$, 10 mM EGTA, 5 mM Na_2ATP, and 10 mM HEPES, with the *p*H adjusted to 7.2 with Tris. The bath solution contained 130 mM tetraethylammonium chloride, 1 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, with the *p*H adjusted to 7.4 with Tris. Whole-cell I_{Ca}s were elicited at 5-s intervals by 150-ms depolarizing pulses (−50 to +40 mV in 10-mV increments) from a holding potential of −70 mV. Inactivation curves were determined using a double-pulse protocol that consisted of a 3-s prepulse to a potential of −70 to +20 mV, followed by a 150-ms depolarization to +20 mV. The peak change in the current during the test pulse was expressed as a fraction of that obtained with the −70-mV prepulse, and this quantity was fit to a Boltzmann expression[17,18] using least-squares analysis to estimate the potential of half-maximal inactivation ($V_{1/2}$) and the slope factor (k).

To measure outward K^+ currents (I_K), recording solutions were chosen to enhance the K^+ currents. The pipette solution contained 70 mM KCl, 60 mM K^+-glutamate, 5 mM K_2ATP, 1 mM $MgCl_2$, 2.5 mM EGTA, 1.8 mM $CaCl_2$, and 10 mM HEPES, with the *p*H adjusted to 7.2 with Tris; the computer-calculated $[Ca^{2+}]_i$ was $\sim 10^{-6}$ M. A variant of this solution contained 10 mM EGTA and no $CaCl_2$, giving a $[Ca^{2+}]_i$ of $\leq 10^{-9}$ M. The bath solution contained 135 mM NaCl, mM KCl 5.2, 1.8 mM $CaCl_2$, 1 mM

$MgCl_2$, 10 mM HEPES, and 10 mM glucose, with the *p*H adjusted to 7.4 with Tris. Whole-cell I_Ks were elicited at 5-s intervals by 150-ms depolarizing pulses (−40 to +60 mV) from a holding potential of −70 mV.

Voltage-pulse protocols were performed in control solutions for more than 5 min to obtain a stable baseline. Cells were exposed to a single concentration of one of the benzodiazepines (diazepam, 10^{-8} to 10^{-3} M; or midazolam, 10^{-8} to 10^{-3} M) tested by changing the inflow perfusate of the chamber to one of a similar composition but with benzodiazepine. The perfusion chamber consisted of a glass coverslip bottom, with needles placed for rapid solution changes. The chamber volume was approximately 1 ml, and complete solution changes in the chamber could be obtained within 1 min using a peristaltic pump (CTP-3; Iuchi, Tokyo, Japan) attached to the input and output ports. After a 6-min exposure, the perfusate was switched again to the control solution. The GΩ seal was maintained for a period sufficient to evaluate the reversibility of the effects of benzodiazepine in 206 of 238 experiments (86%). In another experiment, the effects of the benzodiazepine antagonists flumazenil (10^{-5} M, a specific central type[19]) and PK11195 (10^{-5} M, a specific peripheral type[20,21]) on these channels were tested alone and with these benzodiazepine agonists.

To identify the characteristics of the I_{Ca} seen in this study, the effects of the L-type VDCC antagonist nifedipine (10^{-6} M) and the agonist Bay K 8644 (10^{-6} M) on I_{Ca} were evaluated. The effects of charybdotoxin (40 nM), a specific Ca^{2+}-activated K^+ (K_{Ca}) channel blocker,[22] and 4-aminopyridine (1 mM), a specific Ca^{2+}-independent delayed rectifier K^+ (K_{DR}) channel blocker,[22] on I_K were also evaluated to identify the characteristics of the I_Ks seen in this study.

Materials

The following drugs and chemicals were used: trypsin inhibitor (from soybean), bovine serum albumin, Na_2ATP, pyruvic acid, creatine, taurine, β-hydroxybutyrate, EGTA, TEACl, nifedipine, Bay K 8644, dimethyl sulfoxide, charybdotoxin, 4-aminopyridine (Sigma Chemical Co., St. Louis, MO), type-I collagenase (Gibco Laboratories, Grand Island, NY), protease (Calbiochem, La Jolla, CA), and PK11195 (Research Biochemicals, Natick, MA). Diazepam, midazolam, and flumazenil were donated by Yamanouchi Pharmaceutical Company (Tokyo, Japan). Nifedipine and Bay K 8644 were dissolved in ethanol, and diazepam was dissolved in dimethyl sulfoxide (0.01% final concentrations for both).

Statistical Analysis

Data are expressed as the mean ± SD. The IC_{50}s of the effects of the benzodiazepines on I_{Ca} and I_K were obtained using a Boltzmann expression.[17,18] Changes in peak whole-cell currents (I_{Ca} or I_K) or in the inactivation parameters $V_{1/2}$ and k with exposure to each drug were compared at each applied potential using the paired, two-tailed t test. The percentage of control peak whole-cell currents (I_{Ca} or I_K) and the values of $V_{1/2}$ and k after treatment were compared for the benzodiazepines using one-factor analysis of variance and a Kruskal–Wallis test. In all comparisons, $P < 0.05$ was considered significant.

Results

Electric Properties of Inward Ca^{2+} Currents and the Effects of Benzodiazepines on the the Whole-cell Ca^{2+} Currents

The I_{Ca} seen in enzymatically dispersed canine tracheal smooth muscle cells during step depolarizations from −70 mV peaked at approximately 10 ms and was inactivated with a time constant of approximately 50 to 90 ms (fig. 1A: control). During baseline conditions, threshold activation of I_{Ca} occurred at approximately −20 mV, and maximum peak current amplitude was obtained at approximately +20 mV. In 138 cells, the maximum peak I_{Ca} was −318 ± 26 pA (range, −201 to −612 pA). The inactivation parameters obtained in 28 cells during control conditions were $V_{1/2}$ = −20.4 ± 2.9 mV and k = 7.2 ± 1.3 mV. As previously reported in porcine tracheal smooth muscle cells,[13,14] the addition of 10^{-6} M nifedipine, a blocker of slowly inactivating (L-type) Ca^{2+} channels, virtually eliminated the I_{Ca} of canine tracheal smooth muscle cells by approximately 93%, and 10^{-6} M Bay K 8644, an agonist of L-type Ca^{2+} channels, enhanced the magnitude of I_{Ca} (by approximately 2.4 times) but did not alter the time course of the currents (n = 3 in each case, data not shown). Inward Ca^{2+} currents with a similar time course were observed in the inactivation experiments.

As shown in a representative trace for depolarization from −70 to +20 mV (fig. 1A), midazolam (10^{-4} M) inhibited the magnitude of I_{Ca} but did not obviously alter the time course of the current. Peak I_{Ca} obtained with repeated steps to +20 mV increased in a few minutes after obtaining the whole-cell configuration at time 0 to a stable plateau, decreased rapidly by approximately 50% during exposure to 10^{-4} M midazolam and recovered completely with washout (fig. 1B). Similar results were obtained with diazepam.

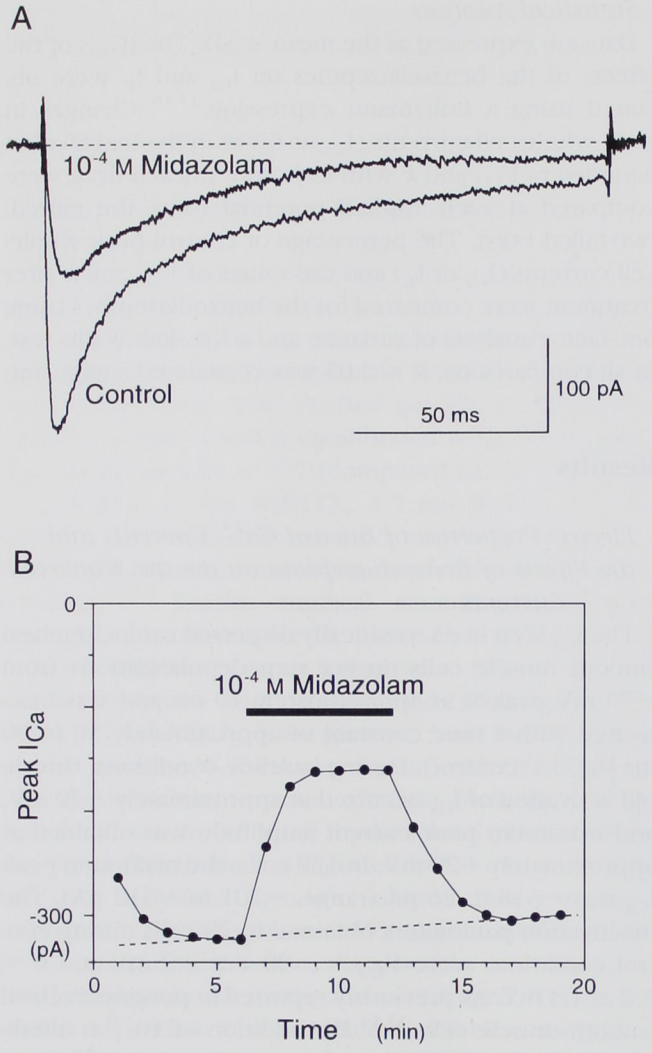

Fig. 1. The effects of midazolam on depolarization-induced whole-cell inward Ca^{2+} current. (*A*) Typical recordings of the whole-cell inward Ca^{2+} current induced by pulses as long as +20 mV in the absence and presence of 10^{-4} M midazolam. The dashed line denotes no current. (*B*) Representative time course of peak I_{Ca} at +20 mV before and after exposure to 10^{-4} M midazolam.

Figure 2 shows the relation between peak I_{Ca} against applied potential before and after exposure to 10^{-6} M diazepam and 10^{-5} M midazolam. Each of these benzodiazepines significantly inhibited I_{Ca} at step potentials ranging from of −10 to +40 mV and decreased peak I_{Ca} at +20 mV by approximately 50% (n = 7). The actual percentage inhibitions of peak I_{Ca} achieved by these agents at these concentrations (50.3 ± 8.4% and 45.8 ± 8.8%, respectively) were not significantly different. There was no apparent shift in the voltage dependence of I_{Ca} with either of the benzodiazepines.

We determined the dose dependence of the inhibition of peak I_{Ca} by each of these benzodiazepines. Figure 3 shows the relation between the percentage of control peak I_{Ca} at +20 mV and the molar concentration of the agents in the bath solution. Each of the two benzodiazepines significantly inhibited peak I_{Ca} in a dose-dependent manner. Midazolam (IC_{50} = approximately 1.2×10^{-5} M) required a 10-fold greater concentration to achieve the same inhibitory effect as that of diazepam (IC_{50} = approximately 10^{-6} M).

Figure 4 and table 1 summarize the effects of the benzodiazepines diazepam and midazolam at equief-

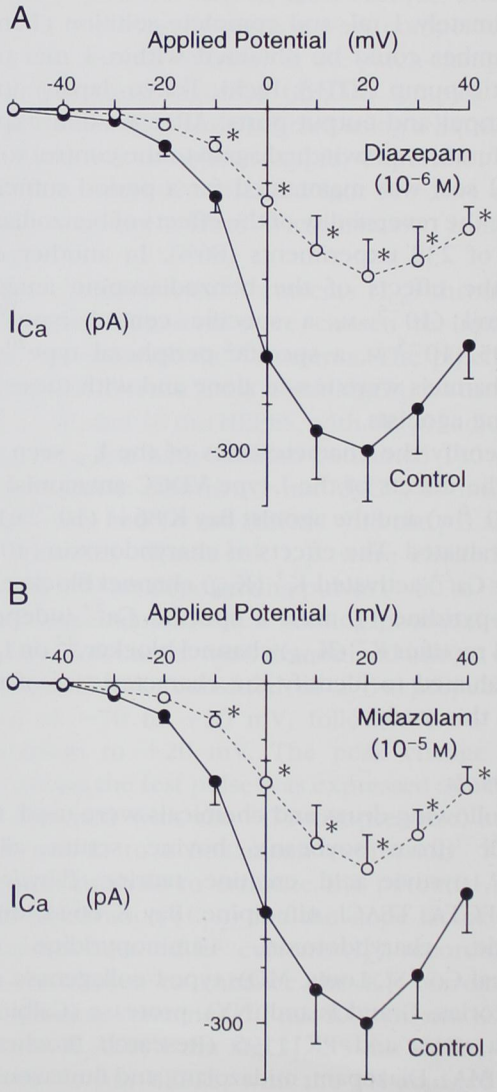

Fig. 2. The relation between the peak whole-cell inward Ca^{2+} current and applied potential before (●, solid line) and after (○, dashed line) exposure to the benzodiazepines 10^{-6} M diazepam (*A*) and 10^{-5} M midazolam (*B*). Symbols represent the mean ± SD (n = 7, *$P < 0.05$).

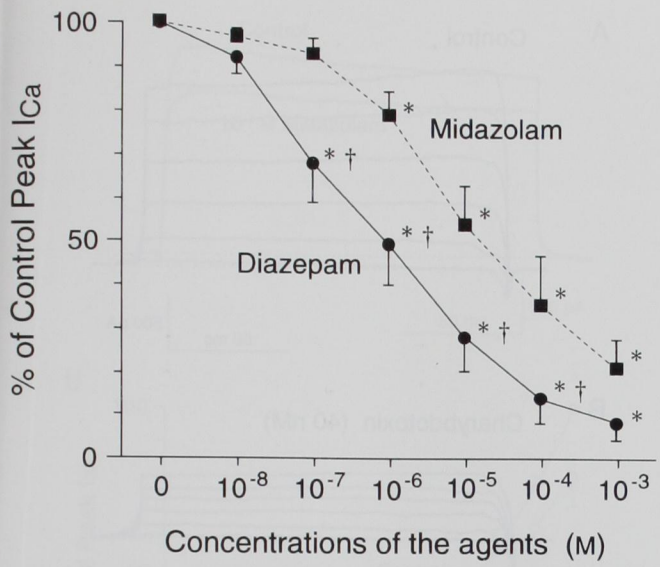

Fig. 3. The relation between peak the whole-cell inward Ca^{2+} current at +20 mV, expressed as a percentage of control, and the bath concentrations of the benzodiazepines diazepam (●, solid line) and midazolam (■, dashed line). Symbols represent the mean ± SD (n = 7). *$P < 0.05$, percentage comparison of control of peak the whole-cell inward Ca^{2+} current without the agents. †$P < 0.05$, comparison of the values of midazolam at the same concentrations.

fective inhibitory concentrations (10^{-6} M and 10^{-5} M, respectively) on the inactivation curves of I_{Ca}. Each of these agents shifted the inactivation curve to a more negative potential. The induced changes in $V_{1/2}$ brought about by these agents were statistically significant in each case, and there was no significant difference in $V_{1/2}$ between these agents. The slope factor k was not changed by exposure to either of the benzodiazepines.

The effects of the benzodiazepine antagonists flumazenil and PK11195 were also tested on the control I_{Ca} and on the inhibitory effect on I_{Ca} of the benzodiazepine agonists diazepam and midazolam. Flumazenil (10^{-5} M) and PK11195 (10^{-5} M) had no significant effect on the control I_{Ca} (n = 3 in each case, data not shown). Figure 5 shows the time course of the peak I_{Ca} obtained in a representative cell with repeated steps to +20 mV during exposure to 10^{-5} M flumazenil and 10^{-6} M diazepam. Despite pretreatment with a high concentration of flumazenil, 10^{-6} M diazepam still induced an approximate 50% inhibition. Similar results were obtained with 10^{-5} M PK11195 and 10^{-6} M diazepam, with 10^{-5} M flumazenil and 10^{-5} M midazolam, and with 10^{-5} M PK11195 and 10^{-5} M midazolam (n = 3 in each case, data not shown).

Electric Properties of Outward K^+ Currents and the Effects of Benzodiazepines on Them

Figure 6A shows a macroscopic outward K^+ current (I_K) obtained from a freshly dispersed canine tracheal smooth muscle cell dialyzed with a pipette solution containing a $[Ca^{2+}]_i$ of $\sim 10^{-6}$ M to enhance I_K through K_{Ca} channels. The I_K was activated progressively by 150-ms depolarizing pulses from a holding potential of −70 mV to consecutively more positive membrane potentials. Stepwise depolarization from a holding potential of −70 mV to more than −30 mV elicited an outward I_K with a mean peak amplitude of 1,840 ± 201 pA at +60 mV (n = 92). The addition of 40 nM charybdotoxin, a specific K_{Ca} channel blocker, significantly decreased peak I_K without any change in the time course of the

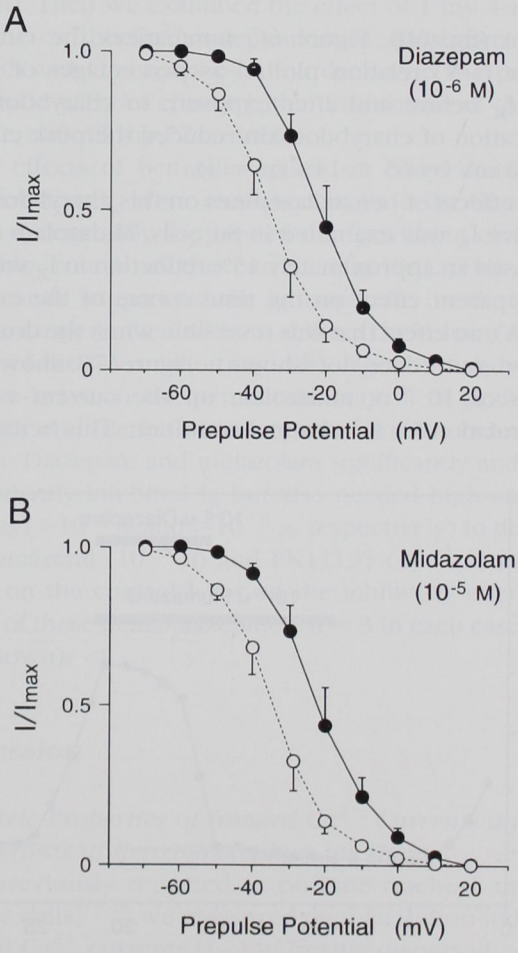

Fig. 4. The effects of the benzodiazepines diazepam (*A*) and midazolam (*B*) on voltage-dependent steady state inactivation of the whole-cell inward Ca^{2+} current. The inactivation curves were generated under control conditions (●, solid line) and then repeated in the presence of one of the benzodiazepines (○, dashed line). Symbols represent the mean ± SD (n = 7).

Table 1 Effects of the Benzodiazepines Diazepam and Midazolam on the Inactivation Parameters of Whole-cell Inward Ca^{2+} Currents (I_{Ca})

	Diazepam (10^{-6} M)	Midazolam (10^{-5} M)
Percent inhibition of peak I_{Ca} at +20 mV	50.3 ± 8.4	45.8 ± 8.8
Potential of half-inactivation ($V_{1/2}$, mV)		
Control	−20.2 ± 3.6	−20.6 ± 2.9
Benzodiazepine	−33.5 ± 3.7*	−32.7 ± 4.0*
Slope factor (k, mV)		
Control	7.1 ± 0.7	7.4 ± 0.8
Benzodiazepine	6.7 ± 1.0	7.2 ± 0.9

Data are expressed as mean ± SD (n = 7). Data were obtained using a 3-s duration prepulse.

* $P < 0.05$, t test comparison to control.

current (fig. 6B). Figure 6C summarizes the current-voltage (I-V) relation plotted as percentages of maximum I_K before and after exposure to charybdotoxin. Application of charybdotoxin reduced the peak current at +60 mV by 65 ± 14% (n = 4).

The effects of benzodiazepines on this charybdotoxin-sensitive I_K was examined in 84 cells. Midazolam (10^{-4} M) caused an approximately 15% reduction in I_K without any apparent effect on the time course of the current (fig. 7A), an effect that was reversible when the drug was washed out (data not shown). Figure 7B shows the effects of 10^{-4} M midazolam on the current-voltage (I-V) relation for K^+ channel activation. This benzodiaz-

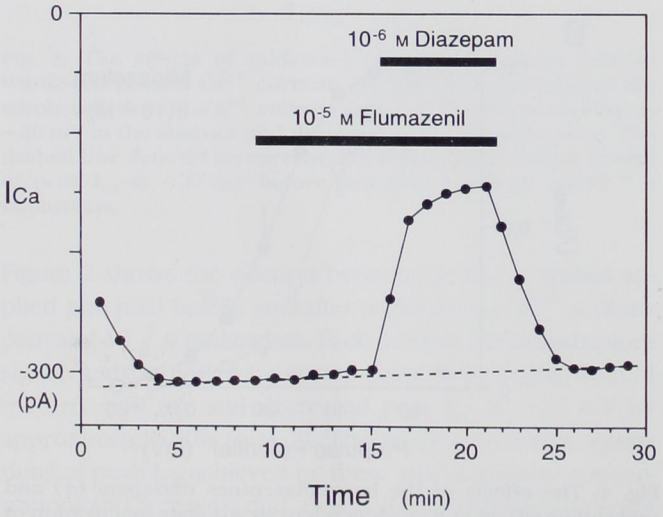

Fig. 5. A representative time course of the peak whole-cell inward Ca^{2+} current obtained with repeated steps to +10 mV during sequential additions of 10^{-5} M flumazenil and 10^{-6} M diazepam.

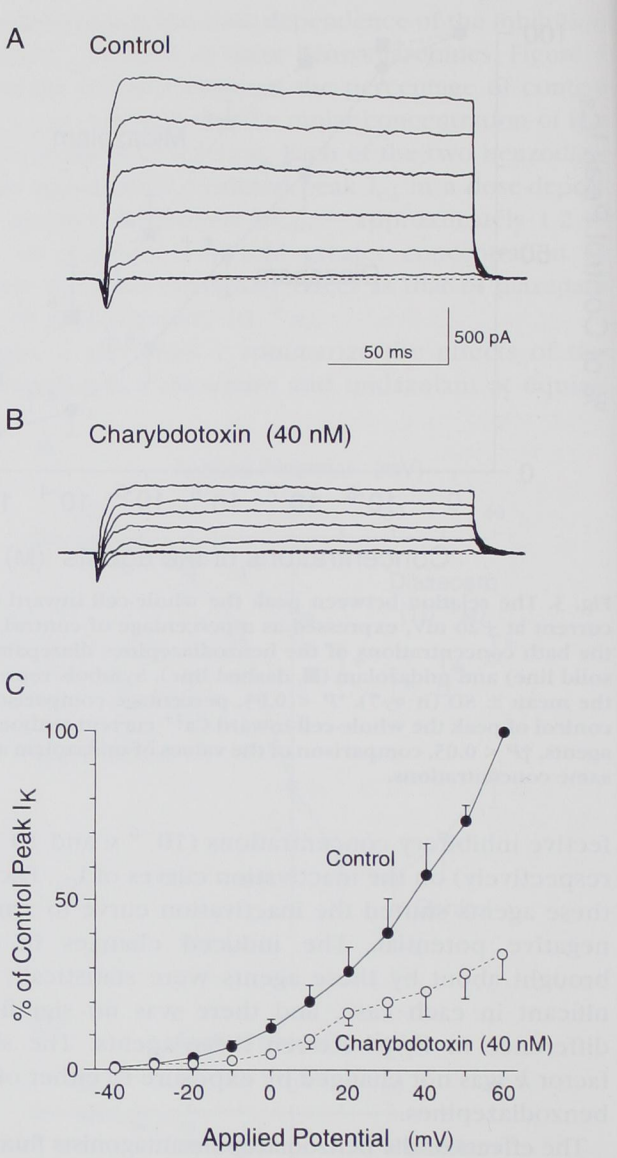

Fig. 6. The whole-cell outward K^+ current in a canine tracheal smooth muscle cell dialyzed with 1.8 mM $CaCl_2$ and 2.5 mM EGTA before (*A*) and after (*B*) exposure to 40 nM charybdotoxin. The The whole-cell outward K^+ currents were generated by depolarizing pulses to −40, −20, 0, +20, +40, and +60 mV from a holding potential of −70 mV. (*C*) Relative peak current–voltage relations obtained before and after exposure to 40 nM charybdotoxin. Symbols represent the mean ± SD (n = 4).

epine significantly suppressed the I_K amplitude over the entire voltage range studied without shifting the voltage dependency of the I-V relation. Figure 7C shows the relation between peak I_K at +60 mV, expressed as a percentage control, and the bath concentrations of the benzodiazepines. Both the benzodiazepines diazepam and midazolam significantly and dose dependently inhibited I_K but required high concentrations of more than

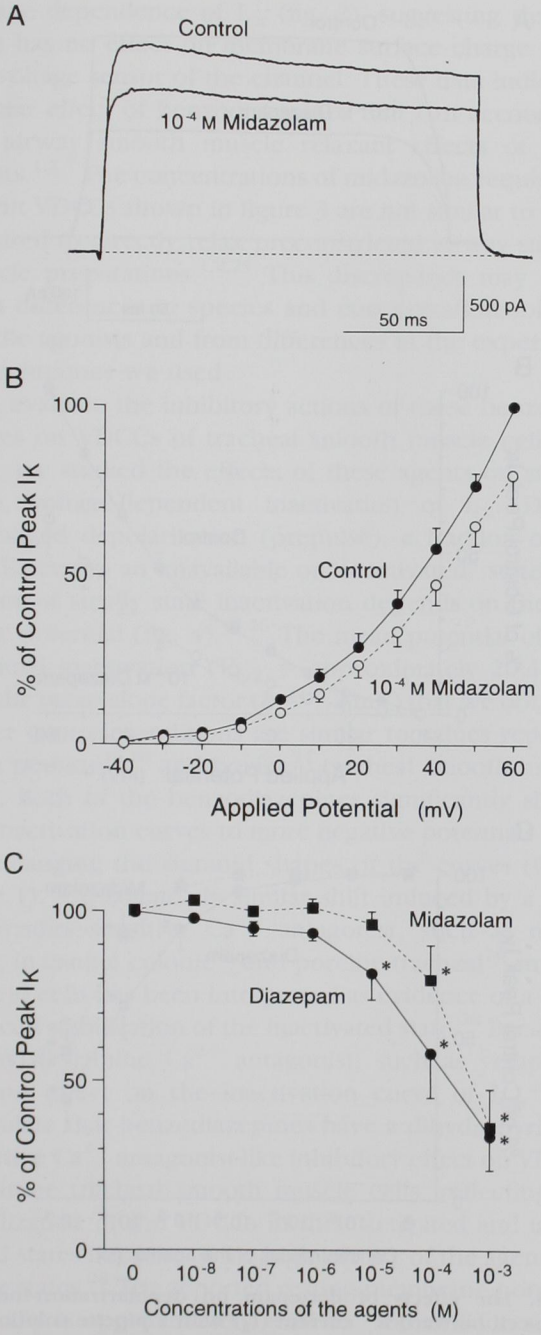

Fig. 7. The effects of midazolam on depolarization-induced whole-cell outward K^+ currents (I_K) with a pipette solution including 1.8 mM $CaCl_2$ and 2.5 mM EGTA. (*A*) Typical recordings of I_K induced by pulses up to +60 mV in the absence and presence of 10^{-4} M midazolam. The dashed line denotes no current. (*B*) Relative peak current-voltage relations obtained before and after exposure to 10^{-4} M midazolam. (*C*) The relation between peak I_K at +60 mV, expressed as a percentage of control, and the bath concentrations of the benzodiazepines diazepam (●, solid line) and midazolam (■, dashed line). Symbols represent the mean ± SD (n = 7). *$P < 0.05$, percentage comparison of control of peak I_K without agents.

10^{-5} M and more than 10^{-4} M, respectively, to show significant effects. Flumazenil (10^{-5} M) and PK11195 (10^{-5} M) had no effect on the control I_K or on the inhibitory effect on the I_K of these benzodiazepines (n = 3 in each case, data not shown).

In a separate series of experiments, we used a pipette solution in which $[Ca^{2+}]_i$ was strongly buffered with 10 ma EGTA to minimize the outward I_K through K_{Ca} channels, and we examined the effects of benzodiazepines on Ca^{2+}-independent I_K. Figure 8A shows a representative trace of I_K under these conditions. The I_Ks were activated progressively by 150-ms depolarizing pulses from a holding potential of −70 mV to consecutively more positive potentials. The mean peak amplitude in 92 cells was 518 ± 110 pA at +60 mV. The application of 40 nM charybdotoxin had no effect on the I_K (n = 3, data not shown). Then we examined the effect of 1 mM 4-aminopyridine on this Ca^{2+}-independent I_K. As shown in figures 8B and C, 4-aminopyridine decreased the peak I_K amplitude without changing the time course of the current at +60 mV by 72 ± 14%.

The effects of benzodiazepines on this 4-aminopyridine-sensitive I_K were examined in 84 cells. Diazepam (10^{-4} M) reversibly suppressed the I_K without changing the time course of the current (fig. 9A). This agent significantly suppressed the I_K amplitude for the entire voltage range studied without shifting the voltage dependency of the I-V relation (fig. 9B). Figure 9C shows the relation between the percentage control of peak I_K at +60 mV and the bath concentrations of the benzodiazepines. Diazepam and midazolam significantly and dose dependently inhibited I_K but also needed high concentrations (>10^{-5} M and >10^{-4} M, respectively) to depress I_K. Flumazenil (10^{-5} M) and PK11195 (10^{-5} M) had no effect on the control I_K or on the inhibitory effects on the I_K of these benzodiazepines (n = 3 in each case, data not shown).

Discussion

Electric Properties of Inward Ca^{2+} Currents and the Effects of Benzodiazepines on Them

As previously reported in porcine tracheal smooth muscle cells,[13,14] we measured depolarization-induced inward Ca^{2+} currents (I_{Ca}) in freshly dispersed canine tracheal smooth muscle cells under ionic conditions designed to inhibit K^+ and Na^+ currents and to enhance Ca^{2+} currents. Based on their time and voltage dependencies, their sensitivity to a nifedipine block-

Interactions between Benzodiazepine Agonists and Antagonists on Whole-cell Inward Ca^{2+} and Outward K^+ Currents

Airway smooth muscle tone also could be regulated by some neuropeptides.[37] γ-Aminobutyric acid (GABA) has an inhibitory effect on postganglionic cholinergic neurotransmission in ferret airways.[38] The benzodiazepine receptor is a positive modulatory subunit of the GABA receptor and enhances the chloride channel currents by increasing its opening frequency.[39] Therefore, in addition to directly inhibiting VDCCs, benzodiazepines might inhibit airway smooth muscle contraction by stimulating some benzodiazepine receptor, which leads to GABA receptor activation. In the current study, however, 10^{-5} M flumazenil and 10^{-5} M PK11195 (specific central[19] and specific peripheral[20,21] benzodiazepine antagonists, respectively) had no effect on the control I_{Ca} and I_K or on the changes in I_{Ca} and I_K induced by the benzodiazepine agonists (fig. 5). Therefore, the diazepam and midazolam benzodiazepines probably relax the airway smooth muscle by binding cell membranes relating to VDCCs, rather than by activating benzodiazepine receptors. In support of our findings, studies have shown that flumazenil and PK11195 have no effect on benzodiazepine-induced relaxation of airway smooth muscle.[1,2,7] The concentration of flumazenil (10^{-5} M) used in this study is greater than the estimated levels of plasma concentrations used clinically.[20,40,41]

Concentration Dependence and Clinical Relevance

The benzodiazepines tested showed dose-dependent inhibition of I_{Ca} and I_K (figs. 3, 7, and 9). Diazepam is more potent than midazolam in terms of I_{Ca} and I_K. Our data should be extrapolated to the clinical situation cautiously because of possible species differences, *in vivo* and *in vitro* differences, and the fact that our patch-clamp experiments were performed at low, nonphysiologic (ambient) temperature and using intracellular (pipette) and extracellular (organ bath) electrolytes. Nonetheless, the plasma concentrations of the benzodiazepines used clinically are approximately 3×10^{-7} to 10^{-5} M.[42–44] In the current study, the bath concentrations of the benzodiazepines diazepam and midazolam used to induce 50% inhibition of I_{Ca} were approximately 10^{-6} M and 10^{-5} M, respectively, which seems relevant to clinical concentrations. We must note, however, that these agents are highly bound to plasma protein (> 90% bound),[42] and the estimated plasma concentrations of free agents seem to be approximately 10^{-9} to 10^{-6} M. Therefore, the similarity between the clinical concentrations[42–44] and the concentrations needed to inhibit I_{Ca} by approximately 50% in this study simply may be a coincidence. Other mechanisms, such that benzodiazepines acts on the medulla to cause airway dilatation,[45] also should be considered.

In conclusion, the benzodiazepines diazepam and midazolam decreased the inward Ca^{2+} current of canine tracheal smooth muscle cells, indicating the inhibition of VDCCs. This response could contribute to the ability of these agents to relax airway smooth muscle *in vitro*. A shift in the inactivation curve by these agents to more negative potentials can be interpreted as evidence of drug-induced stabilization of the inactivated state. Because the benzodiazepines reduced the K^+ channel activity at high concentrations, K^+ channel opening could not be responsible for the mechanisms of benzodiazepine-induced bronchodilation. The lack of antagonized effects of their antagonists flumazenil and PK11195 is related to the non-GABA–mediated electrophysiologic effects of benzodiazepines on airway smooth muscle contractility.

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