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Effects of Intravenous Anesthetics on Ca^{2+} Sensitivity in Canine Tracheal Smooth Muscle

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Background: **Halothane and other volatile anesthetics relax airway smooth muscle in part by decreasing the amount of force produced for a particular intracellular calcium concentration (the Ca^{2+} sensitivity) during muscarinic receptor stimulation. In this study, ketamine, propofol, and midazolam were evaluated to determine whether the inhibitory effect of volatile anesthetics on this signal transduction pathway is a general property of other types of anesthetic drugs.**

Methods: **A β-escin permeabilized canine tracheal smooth muscle preparation was used. Ketamine, propofol, and midazolam, in concentrations producing near-maximal relaxation in intact airway smooth muscle (200 μM, 270 μM, and 100 μM, respectively), were applied to permeabilized muscles stimulated with calcium in either the absence or the presence of muscarinic receptor stimulation provided by acetylcholine. The effect of halothane also was evaluated.**

Results: **Confirming previous studies, halothane (0.75 mM) decreased calcium sensitivity during muscarinic receptor stimulation. None of the intravenous anesthetics studied affected Ca^{2+} sensitivity, either in the absence or the presence of muscarinic receptor stimulation.**

Conclusions: **Intravenous anesthetics in high concentrations directly relax canine tracheal smooth muscle without affecting Ca^{2+} sensitivity. The inhibition of agonist-induced increases in Ca^{2+} sensitivity of canine tracheal smooth is not a common property of anesthetics, but is unique to volatile agents. (Key words: Airways; bronchodilators; G proteins; intracellular calcium concentration.)**

CONTRACTION of smooth muscle in response to physiologic agonists is associated with an increase in the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_i$). However, contractile force is not determined by $[Ca^{2+}]_i$ alone in smooth muscle because membrane receptor stimulation with various receptor agonists increases force developed at a constant $[Ca^{2+}]_i$ (*i.e.,* increases Ca^{2+} sensitivity).[1,2] We showed that halothane directly relaxes airway smooth muscle, not only by decreasing $[Ca^{2+}]_i$,[3] but also by decreasing Ca^{2+} sensitivity during membrane receptor stimulation.[1,4] At the equivalent minimum alveolar concentration (MAC), sevoflurane has a lesser effect on Ca^{2+} sensitivity, and isoflurane has a small, insignificant effect.[5] Thus, at concentrations equipotent for producing anesthesia, volatile anesthetics differ in the ability to inhibit Ca^{2+} sensitivity in airway smooth muscle.

Data concerning the effects of other types of anesthetics on Ca^{2+} sensitivity in airway smooth muscle are few. Three intravenous drugs used clinically to induce anesthesia have been reported to directly relax airway smooth muscle, and thus might inhibit calcium sensitivity. Ketamine and propofol appear to be clinically useful in the treatment of patients with hyper-reactive airways. These agents inhibit neural pathways, innervating smooth muscle, and also directly relax airway and other types of smooth muscle.[6–9] This direct relaxation is associated with a decrease in $[Ca^{2+}]_i$ caused by an inhibition of Ca^{2+} influx.[6,10] It is not known whether these drugs may also affect Ca^{2+} sensitivity. Midazolam also directly relaxes airway smooth muscle and decreases $[Ca^{2+}]_i$;[11,12] however, one study of intact airway smooth muscle found that it does not affect Ca^{2+} sensitivity.[11]

In the current study, we determined the effects of three intravenous anesthetics on Ca^{2+} sensitivity in airway smooth muscle using a permeabilized canine tracheal smooth muscle (CTSM) preparation in which Ca^{2+} sensitivity can be directly assessed.[1] Ketamine, propofol, and midazolam were evaluated to determine whether the previously observed inhibitory effect of halothane on agonist-induced increase in Ca^{2+} sensitivity in airway

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smooth muscle is shared by intravenous anesthetics that also relax airway smooth muscle.

Materials and Methods

Tissue Preparation

After approval by the Institutional Animal Care and Use Committee, mongrel dogs (20–25 kg) of either sex were anesthetized using an intravenous injection of sodium pentobarbital (50 mg/kg) and were killed by exsanguination. A 10- to 15-cm portion of extrathoracic trachea was excised and immersed in chilled physiologic salt solution of the following composition: NaCl: 110.5 mM; $NaHCO_3$: 25.7 mM; dextrose: 5.6 mM; KCl: 3.4 mM; $CaCl_2$: 2.4 mM; KH_2PO_4: 1.2 mM; $MgSO_4$: 0.8 mM. The adventitia and mucosa were removed after cutting the visceral side of the cartilage, then connective tissues were carefully removed during microscopic observation.

Isometric Force Measurements

Muscle strips (width, 0.1–0.2 mm; wet weight, 0.05–0.1 mg) were mounted in 0.1-ml glass cuvettes and continuously superfused at 1.2 ml/min with physiologic salt solution (37°C) aerated with 94% oxygen and 6% carbon dioxide, providing a pH of ≈ 7.4, a partial pressure of oxygen of ≈ 400 mmHg, and a partial pressure of carbon dioxide of ≈ 39 mmHg in the physiologic salt solution. One end of the muscle strips was anchored with stainless steel microforceps to a stationary metal rod, and the other end was anchored to a calibrated force transducer (model KG4; Scientific Instruments, Hedelberg, Germany). The initial gap between microforceps (*i.e.,* initial muscle length) was set at 5 mm. During a 3-h equilibration period, the length of the muscle strips was increased after subsequent isometric contractions (of 1-min duration) induced by 1 μM acetylcholine until isometric force was maximal (optimal length). Each muscle strip was maintained at this optimal length for the remainder of the experiment. These tissues produced maximal isometric forces of 1–3 mN when stimulated with 1 μM acetylcholine.

Permeabilization Procedure

The muscle strips were permeabilized with β-escin, as previously described,[13] and validated in the Mayo Clinic and Mayo Foundation laboratory.[1] β-escin creates pores in the smooth muscle cell plasma membrane, thus allowing substances of small molecular weight, such as Ca^{2+}, to freely diffuse across the cell membrane. Accordingly, $[Ca^{2+}]_i$ can be manipulated and controlled by changing the concentration of Ca^{2+} in the solution that bathes the smooth muscle strip. Larger cellular proteins necessary for contraction are preserved. Additionally, the membrane receptor–coupled mechanisms that modulate Ca^{2+} sensitivity remain intact and can be activated.[1]

After optimal length was set, subsequent experimental protocols were performed at room temperature (25°C) and without aeration of the solutions. Muscle strips were superfused for 20 min, with a relaxing solution that contained 100 μM β-escin. The relaxing solution was made up in the following composition using the algorithm of Fabiato and Fabiato:[14] 7.5 mM MgATP, 4 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′N′-tetraacetic acid (EGTA), 20 mM imidazole, 1 mM dithiothreitol, 1 mM free Mg^{2+}, 1 nM free Ca^{2+}, 10 mM creatine phosphate, and 0.1 mg/ml creatine phosphokinase. Ionic strength was kept constant at 200 mM by adjusting the concentration of potassium acetate. The pH was adjusted to 7.0 at 25°C with potassium hydroxide. After the permeabilization procedure, strips were superfused with the relaxing solution for 10 min to wash out the excess β-escin. The calcium ionophore A23187 (10 μM) was added to the relaxing solution and all subsequent experimental solutions to deplete the sarcoplasmic reticulum Ca^{2+} stores and maintain $[Ca^{2+}]_i$.[1] Solutions of varying free Ca^{2+} concentrations used in the subsequent experiment also were prepared using the aforementioned algorithm.[14]

Experimental Protocols

Effects of Anesthetics on Acetylcholine-induced Contraction in Intact Canine Tracheal Smooth Muscle. Experiments with intact strips were designed to determine what concentrations of drugs should be used in subsequent studies of permeabilized strips. This protocol was performed at 37°C in physiologic salt solution. Muscle strips were contracted for 10 min with 0.03 μM acetylcholine, which produced approximately 50% of the maximal force induced by 100 μM acetylcholine in preliminary studies (data not shown). After stable contractions were obtained, cumulative doses of ketamine ($10^{-7} - 10^{-3}$ M), propofol ($10^{-6} - 10^{-3}$ M), or midazolam ($10^{-7} - 1.8 \times 10^{-4}$ M) were applied, and concentration–response curves for these anesthetics were generated. The effect of 10% intravenous fat emulsion, similar to the vehicle for propofol, also was evaluated. The effect of halothane on intact CTSM was reported in a previous study.[15]

Effects of Anesthetics on Ca^{2+} Sensitivity in Permeabilized Canine Tracheal Smooth Muscle. Experiments with permeabilized strips determined the effects of anesthetics on the Ca^{2+} sensitivity in the absence and the presence of muscarinic receptor stimulation. A set of four β-escin permeabilized strips was prepared from the same dog for each experiment. All strips first were contracted maximally with 10 μM Ca^{2+}; all subsequent force measurements were normalized to these contractions. Strips were superfused with relaxing solution that contained 5 mM inorganic phosphate for 10 min and then superfused with relaxing solution for 10 min to remove inorganic phosphate. Inorganic phosphate reduces the time necessary for relaxation by accelerating the rate of actomyosin cross-bridge detachment.[16] Each set of four strips was studied as two pairs. All four strips were contracted with 0.3 μM Ca^{2+} for 10 min. One pair was then stimulated with 10 μM acetylcholine and 10 μM guanosine 5′-triphosphate; the other pair continued to be exposed to Ca^{2+} alone. After 10 min, one of four drugs (one drug for each set of muscles) was added to one strip of each pair for 15 min. For ketamine, propofol, and midazolam, an approximate EC_{80} concentration was used (200, 270, and 100 μM, respectively). For halothane, a concentration equivalent to approximately 3 MAC (for dogs, corrected to room temperature[17]) was used. The remaining strip of each pair was not exposed to drugs and served as a time control. In experiments with propofol, an equivalent volume of the vehicle 10% intravenous fat emulsion was added at the appropriate time for the time-control strips.

Administration of Halothane. Halothane was delivered to solutions *via* a calibrated vaporizer. Each solution was equilibrated with halothane for at least 5 min (enough time to become equilibrated in our system) before being introduced to the superfusion system. At the end of the protocol, the concentrations of halothane in the solutions at the cuvette were determined by gas chromatography from anaerobically obtained samples using an electron capture detector (model 5880A; Hewlett-Packard, Waltham, MA) according to the method of Van Dyke and Wood.[18]

Materials

Halothane was purchased from Ayerst Laboratories (New York, NY). Adenosine 5′-triphosphate, disodium salt was purchased from Research Organics (Cleveland, OH). Propofol was purchased from Zeneca Pharmaceuticals (Wilmington, DE). Intravenous fat emulsion, 10%, was purchased from Baxter Health Care (Deerfield, IL).

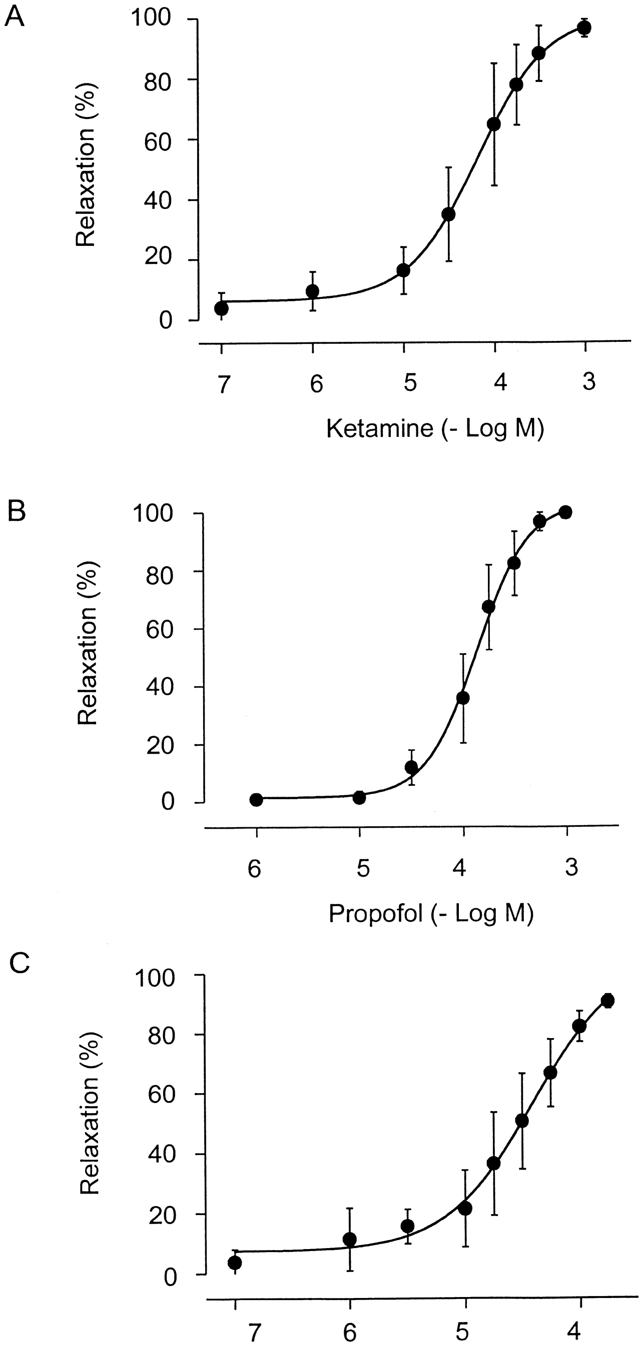

Fig. 1. The effect of intravenous anesthetics on isometric force induced by 0.03 μM acetylcholine in intact canine tracheal smooth muscle (CTSM). Ketamine (*A*), propofol (*B*), and midazolam (*C*) relaxed intact CTSM in a concentration-dependent manner. Values are the mean ± SD (n = 5).

All other drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO). The calcium ionophore A23187 was dissolved in dimethyl sulfoxide (0.05% final concentration). Propofol was diluted from an aqueous emulsion (5.6×10^{-2} M) in 10% (wt/vol) soybean oil, 2.25% glycerol, and 1.2% purified egg lecithin. All other drugs and chemicals were prepared in distilled filtered water.

Statistical Analysis

Data are expressed as the mean ± SD; n represents the number of dogs. Concentration–response curves for ketamine, propofol, and midazolam were fitted by nonlinear regression (SigmaPlot for Windows Version 4.00; SPSS Inc., Chicago, IL) and used to calculate EC_{80} values.

In the first protocol, relaxation produced by anesthetics was expressed as a percent change from the initial force produced by 0.03 μM acetylcholine. In the second protocol, forces were expressed as a percentage of the maximal force induced by 10 μM Ca^{2+} determined in each individual strip before the experimental protocol. The decrease in force produced by anesthetics was expressed as a percent relaxation from initial force (before exposure to drug). Initial force was adjusted for the effect of time by using the change in force of the time-matched control strip in each set according to the following formula:

$$\%\text{relaxation} = (1 - (A_2/A_1) \times (C_1/C_2)) \times 100$$

where A_1 is the force of the anesthetic-exposed strip just before exposure to anesthetic, A_2 is the force of the anesthetic-exposed strip at the end of anesthetic exposure, C_1 and C_2 are the forces of the control strip at the matched times with A_1 and A_2, respectively. All force values represent a change from baseline force (*i.e.,* in relaxing solution). Statistical assessments were made using the paired *t* test. A *P* value < 0.05 was considered to be statistically significant.

Results

Effects of Anesthetics on Acetylcholine-induced Contraction in Intact Canine Tracheal Smooth Muscle

Ketamine, propofol, and midazolam all produced concentration-dependent relaxation of intact CTSM strips contracted with acetylcholine (figs. 1A–C). EC_{80} values for this effect were 199, 274, and 99 μM for ketamine, propofol, and midazolam, respectively. The vehicle for propofol, 10% intravenous fat emulsion, did not affect contraction (data not shown).

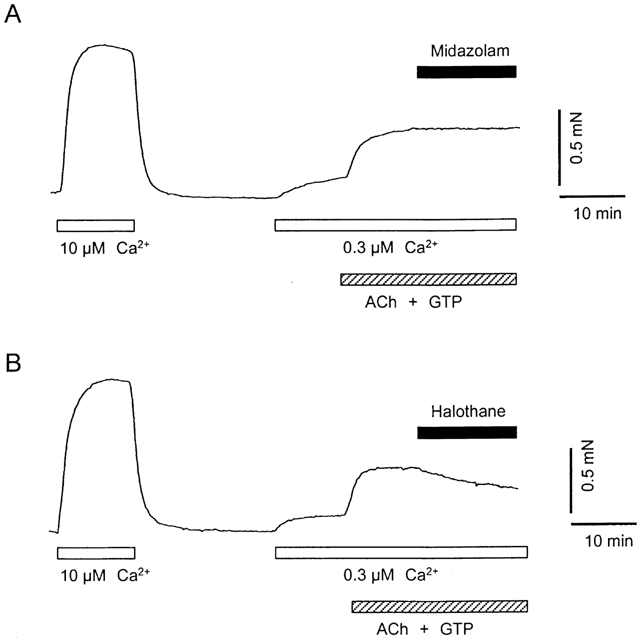

Fig. 2. Representative recordings of the application of midazolam (*A*), and halothane (*B*) on acetylcholine-induced Ca^{2+} sensitization in β-escin–permeabilized canine tracheal smooth muscle. Muscarinic receptor stimulation with 10 μM acetylcholine (ACh) plus 10 μM guanosine 5′-triphosphate (GTP) increased force during exposure to 0.3 μM Ca^{2+} (indicating an increase in Ca^{2+} sensitivity). Note that halothane, but not midazolam, decreased calcium sensitivity.

Effects of Anesthetics on Ca^{2+} Sensitivity in Permeabilized Canine Tracheal Smooth Muscle

In β-escin–permeabilized tissue, 0.3 μM Ca^{2+} induced stable contractions of 7.4 ± 5.5% (n = 80 strips) of the maximal force induced by 10 μM Ca^{2+} (fig. 2). The subsequent addition of 10 μM acetylcholine and 10 μM guanosine 5′-triphosphate further increased the force to 38.7 ± 10.4% of maximal force after 10 min, indicating an increase in Ca^{2+} sensitivity produced by acetylcholine and guanosine 5′-triphosphate. None of the three intravenous anesthetics studied significantly changed force, either in the presence or the absence of receptor stimulation with acetylcholine (figs. 2 and 3). Consistent with our previous work,[1] halothane (0.75 ± 0.05 mM) significantly reduced force in strips stimulated with acetylcholine (by 27.8 ± 13.4%) but did not change force in strips stimulated only with Ca^{2+} (figs. 2 and 3).

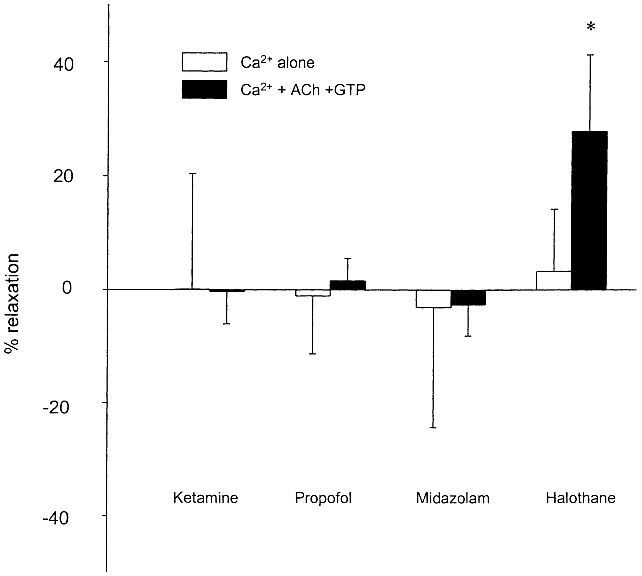

Fig. 3. The effect of intravenous anesthetics and halothane on isometric force induced by 0.3 μM Ca^{2+} in the absence (open columns) and presence (filled columns) of muscarinic receptor stimulation with 10 μM acetylcholine (ACh) and 10 μM guanosine 5′-triphosphate (GTP). Data are expressed as percent relaxation corrected for time (see text). Values are the mean ± SD ($n = 5$). *Significant difference from 0, $P < 0.05$.

Discussion

The major finding of this study in β-escin–permeabilized CTSM is that ketamine, propofol, or midazolam did not affect Ca^{2+} sensitivity, either in the presence or the absence of muscarinic receptor stimulation. Therefore, the effect of halothane to inhibit agonist-induced increases in Ca^{2+} sensitivity, confirmed in this study, is not a general feature of drugs with anesthetic properties.

Many anesthetic agents act as bronchodilators *in vivo*.[19,20] These drugs depress neural transmission in pathways that innervate airway smooth muscle and also directly affect the smooth muscle cell. In general, these direct effects are caused by a decrease in $[Ca^{2+}]_i$, a decrease in the force maintained for a particular $[Ca^{2+}]_i$ (*i.e.,* the Ca^{2+} sensitivity), or a combination of both mechanisms. It is clear that during submaximal contraction of airway and other types of smooth muscle, anesthetic-induced relaxation is associated with a decrease in $[Ca^{2+}]_i$. This is true for the volatile anesthetics[3,21,22] and for the three intravenous anesthetics evaluated in our study: ketamine,[7,10] propofol,[10] and midazolam.[11] For these intravenous anesthetics, decreases in $[Ca^{2+}]_i$ are caused by inhibition of Ca^{2+} influx through L-type voltage-operated Ca^{2+} channels.[6,10] Although volatile anesthetics may affect intracellular Ca^{2+} stores,[3] these intravenous anesthetics do not appear to do so in studies to date.[9,11] The barbiturates, the other major class of intravenous induction agents used clinically, also may depress airway neural transmission in intact animals but, in most recent studies, do not affect or actually cause contraction of isolated airway smooth muscle.[23,24] In preliminary studies, we found little effect of thiopental on canine airway smooth muscle (data not shown) and therefore elected not to study this agent further.

Although it is clear that anesthetics relax airway smooth muscle in part by decreasing $[Ca^{2+}]_i$, inhibition of Ca^{2+} sensitivity also may be important. We evaluated Ca^{2+} sensitivity by using a β-escin–permeabilized smooth muscle preparation. The creation of pores in the plasma membrane permits the manipulation of the intracellular environment by changing the composition of the fluid that bathes the smooth muscle, such that Ca^{2+} sensitivity may be directly studied.[1,13] Contraction of this preparation is produced by exposing it to Ca^{2+}; exposure to muscarinic receptor agonists produces additional force (fig. 2). The binding of Ca^{2+} to calmodulin increases myosin light chain kinase activity and phosphorylation of the 20-kd regulatory myosin light chain (rMLC).[25,26] Regulatory myosin light chain phosphorylation allows the binding of myosin to actin, which increases actomyosin adenosine triphosphatase activity and causes contraction.[2] Muscarinic receptor stimulation activates a cascade of G proteins that inhibit smooth muscle protein phosphatases, increasing regulatory myosin light chain phosphorylation and, thus, force.[27,28] A previous study from our laboratory showed that permeabilization of CTSM with β-escin eliminates Ca^{2+} gradients across the sarcolemma and maintains $[Ca^{2+}]_i$ during muscarinic receptor stimulation and that the coupling of membrane receptors to mechanisms that increase Ca^{2+} sensitivity remain intact and can be activated.[1]

We confirmed our previous finding that halothane significantly decreases Ca^{2+} sensitivity during muscarinic receptor stimulation of the permeabilized preparation exposed to a submaximal concentration of Ca^{2+}.[1] However, none of the three intravenous anesthetics studied (at approximate ED_{80} concentrations for relaxation of intact muscle) significantly affected the force produced by constant submaximal $[Ca^{2+}]_i$, either in the presence or the absence of muscarinic receptor stimulation. Yoshimura *et al.*[11] also found no effect of midazolam on Ca^{2+} sensitivity measured in an intact CTSM preparation. Taken together with previous studies of the effects of these drugs on $[Ca^{2+}]_i$, we conclude that, at concentrations producing near-maximal effect, these drugs relax

CTSM exclusively by decreasing $[Ca^{2+}]_i$, without affecting Ca^{2+} sensitivity. Although comparisons should be made with caution, these ED_{80} concentrations of drugs considerably exceed the reported plasma concentrations that produce surgical anesthesia in humans (approximately 2- 60 μM,[29] 20-50 μM,[30,31] and 1-2 μM[32] for ketamine, propofol, and midazolam, respectively). When the fact that these drugs are significantly protein-bound ($>$ 95% for propofol and midazolam)[30,31,33] is also taken into account, it is almost certain that they would not affect Ca^{2+} sensitivity as clinically used.

In conclusion, three intravenous anesthetics (ketamine, propofol, and midazolam) directly relax canine airway smooth muscle stimulated with a muscarinic agonist without affecting Ca^{2+} sensitivity. Thus, inhibition of agonist-induced increases in Ca^{2+} sensitivity of CTSM is not a common property of anesthetics but is unique to the volatile agents.

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