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Halothane and Isoflurane Do Not Directly Interact with Cardiac Cross-bridge Function

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Background: **Halogenated anesthetics depress myocardial contractility by altering a number of specific mechanisms. These alterations include decreases in inward calcium current and sarcoplasmic reticulum function and reduced calcium myofilament sensitivity. However, the direct effects of volatile anesthetics on cross-bridge function have yet to be precisely determined.**

Methods: **Myosin monomers and actin filaments were isolated from fresh rat left ventricles and rabbit skeletal muscles, respectively. Halothane or isoflurane was added at concentrations equivalent to 1 and 2 minimum alveolar concentration (MAC). Motility of actin filaments over myosin was initiated by adding 2 mM adenosine triphosphate and was analyzed at 30°C. Maximum actomyosin adenosine triphosphatase activity and the association constant of myosin for actin were determined from a double-reciprocal Lineweaver–Burk plot of the adenosine triphosphatase rate *versus* actin concentration. A known inhibitor of actomyosin function, 2,3-butanedione 2-monoxime (2 mM), was used in positive control experiments. Data are presented as mean ± SD.**

Results: **Motility velocities driven by myosin were not significantly different between baseline and 1 and 2 MAC halothane (2.70 ± 0.33, 2.72 ± 0.36, and 2.70 ± 0.40 μm/s, respectively). Similarly, motility velocities driven by myosin were not significantly different between baseline and 1 and 2 MAC isoflurane (2.73 ± 0.33, 2.72 ± 0.37, and 2.72 ± 0.40 μm/s, respectively). Neither of the two halogenated anesthetics, at any concentration tested, significantly modified the maximum actomyosin adenosine triphosphatase activity or the association constant of myosin for actin as compared with baseline. 2,3-Butanedione 2-monoxime induced a drastic reduction in both motility velocity and maximum actomyosin adenosine triphosphatase activity.**

Conclusion: **These results indicate that isoflurane and halothane do not directly depress the mechanical or enzymatic properties of cross-bridges in the heart.**

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VOLATILE halogenated anesthetics are known to depress myocardial contractility both *in vivo* and *in vitro* in a dose-dependent manner.[1,2] Negative inotropic effects mainly result from a decrease in myoplasmic Ca^{2+} available for contraction, related to reduced inward calcium current and sarcoplasmic reticulum function and to a decreased responsiveness of myofilament sensitivity to Ca^{2+}.[3–6] In addition, it has been proposed that halogenated anesthetics modulate cross-bridge cycling in both intact[7,8] and skinned[9–12] cardiac muscles. These mechanical changes are associated with enzymatic changes in myosin properties, although certain authors report decreased actin-activated adenosine triphosphatase (ATPase) activity of myosin,[13–16] whereas others report an increase in this activity.[17] However, the significance of the results of these investigations is difficult to determine because the anesthetic concentrations studied were high,[13–16] the myosin ATPase was not physiologically activated,[18] or both. In addition, because of their many sites of action, direct effects of halogenated anesthetics on cross-bridge function are difficult to analyze in cardiac muscle. Changes in the kinetics and number of active cross-bridges may result from changes in Ca^{2+} homeostasis, the affinity of troponin C for Ca^{2+} under the effect of halogenated anesthetics, or both, thereby leading to misinterpretation. Alternatively, local anesthetics have been shown to directly inhibit actomyosin motility *in vitro* without affecting the actomyosin ATPase activity.[19] Whether halogenated anesthetics exert similar effects on purified myosin molecules remains to be determined.

In this *in vitro* study, we investigated the effects of clinically relevant concentrations (1 and 2 minimum alveolar concentration [MAC]) of halothane and isoflurane on both the mechanical and enzymatic properties of cardiac myosin molecules. Our aim was to determine whether the potential effects of halogenated anesthetics were related to direct inhibition of cardiac myosin function. Furthermore, 2,3-butanedione 2-monoxime (BDM), a well-known inhibitor of actomyosin function,[20,21] was used to validate the sensitivity of our preparation to drugs.

Materials and Methods

Care of the animals conformed to the recommendations of the Helsinki Declaration, and the study was performed in accordance with the regulations of the official edict of the French Ministry of Agriculture. These experiments were conducted in an authorized laboratory under the supervision of authorized researchers

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(B. R. and C. C.). Experiments were conducted in adult male Sprague-Dawley rats obtained from Charles River Laboratories (L'Arbresle, France). After anesthesia with intraperitoneal pentobarbital sodium (60 mg/kg), the heart was removed, and the left ventricle was immediately dissected.

Preparation of Contractile Proteins

Myosin isolated from fresh left ventricle was homogenized in high-salt buffer (1:5 weight:volume; 0.3 M KCl, 150 mM K_2HPO_4, 10 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 150 mM imidazole, and 1 mM adenosine triphosphate [ATP]; pH 6.8) for 20 min.[22,23] The homogenate was centrifuged at 150,000*g* for 45 min at 4°C with Beckman TLC 100 (Beckman Coulter France, Roissy CDG, France). The supernatant was diluted by 20 times or more in 2 mM DTT and 1 mM ATP and was allowed to stand on ice for 30 min to precipitate filamentous myosin. Myosin was then collected by centrifugation at 80,000*g* for 15 min in Beckman Ti70. The pellet was dissolved in myosin buffer (300 mM KCl, 25 mM imidazole, 4 mM $MgCl_2$, 1 mM DTT, and 1 mM EGTA; pH 7.4). The purity of myosin extraction was assessed using electrophoresis analysis. Fresh myosin was used within 48 h. Actin was prepared from rabbit skeletal muscle[24] and was fluorescently labeled with tetramethylrhodamine-phalloidin (Molecular Probes, Eugene, OR).

In Vitro *Motility Assays*

In vitro motility assays were performed at 30°C as previously described.[25,26] In brief, a microscope flow cell was constructed from a nitrocellulose-coated coverslip, two glass spacers, and a glass microscope slide.[27] The protein samples and buffer solutions were infused into the microscope flow cell at 90-s intervals in the following order. First, the myosin sample was diluted to 100 μg/ml in myosin buffer and applied to the flow cell. Unbound myosin was then washed out with high-salt myosin buffer: 0.5 mg/ml bovine serum albumin. Actin buffer consisting of 25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM $MgCl_2$, and 10 mM DTT was then infused into the flow cell with 0.5 mg/ml bovine serum albumin. To block nonfunctional myosin molecules that could bind actin filaments but not detach from them in the presence of ATP, unlabeled F-actin filaments in low-salt buffer were applied to the flow cell. To remove F-actin from functional myosin heads, actin buffer containing 2 mmol ATP was applied, followed by actin buffer. Next, fluorescent actin filaments in actin buffer were added at a concentration of 80 ng/ml. Unbound actin was washed out by actin buffer with an oxygen-scavenger enzyme system (20 mmol DTT, 0.02 mg/ml catalase, 0.1 mg/ml glucose oxidase, and 3 mg/ml glucose). Solutions of halothane or isoflurane were added to the oxygen-scavenger enzyme to obtain 250 and 500 μM. These concentrations are equivalent to 1 and 2 MAC of halothane and isoflurane in the adult rat at 30°C.[28] In addition, in a subgroup of experiments without volatile anesthetics, BDM was added to the oxygen-scavenger enzyme to obtain a final concentration of 2 mM.[20,21] In each case, motility of actin filaments was initiated by adding ATP at a concentration of 2 mM. The movement of actin filaments was observed after a stabilization period of 5 min under an epifluorescence microscope (Axiovert 200, 100/1.30 lens; Zeiss, Jena, Germany) with an intensified camera (Hamamatsu C 2400; Hamamatsu City, Japan) and recorded on videotape (additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org). A stabilization period of 5 min was performed after the addition of drugs, ATP, or both. Such delay is not expected to be associated with a significant decrease in anesthetic concentration.[29] Actin filament movements over myosins were analyzed when more than 85% of all filaments were moving continuously within a visual field. Filament velocities were analyzed using N. J. Carter's freeware RETRAC program, *i.e.*, a mouse-based tracking multiple moving objects in a sequence of images that provides velocity and direction statistics from sliding actin filaments.‖

ATPase Assays

Actin-activated ATPase activity was determined at 21°C according to the method of White.[30] Briefly, myosin was diluted to 0.1 mg/ml in actin buffer. Actin was added to final concentrations of 0, 5, 10, 30, and 60 μM, and the reaction was started with 2 mM MgATP. Experiments were performed at baseline, after the addition of 2 mM BDM, or after the addition of halothane or isoflurane at concentrations equivalent to 1 and 2 MAC. Inorganic phosphate concentrations were determined colorimetrically at fixed time points. Observed ATPase rates were corrected for ATP hydrolysis in the absence of actin. The maximum actomyosin ATPase activity (Vmax, s^{-1}) and the association constant of myosin for actin (Km, μmol) were determined from a double-reciprocal Lineweaver–Burk plot of the ATPase rate *versus* actin concentration.

Statistical Analysis

Data are expressed as mean ± SD. The mean velocity was calculated for each filament. For each group, ATPase activity measurements were repeated at least five times. The effects of halogenated anesthetics or BDM on filament velocities and enzymatic properties were assessed by using analysis of variance. In addition, the Kolmogorov–Smirnov test was used to determine difference between velocity distributions. All *P* values were two-tailed and a *P* value of less than 0.05 was required to rule out the null hypothesis. Statistical analysis was performed

‖ Available at: http://mc11.mcri.ac.uk/Retrac. Accessed October 26, 2004. Dr. Nick Carter, PhD, Senior Research Associate, Marie Curie Research Institute, Molecular Motor Group, The Chart, Oxted, Surrey, United Kingdom.

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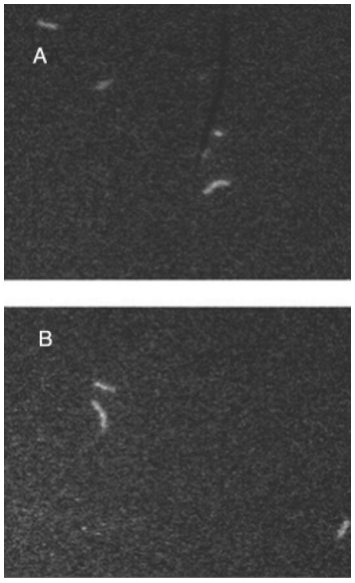

Fig. 1. Typical images of actin filament sliding over myosin at baseline (*A*) and with isoflurane (*B*). Additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org.

using Statview software version F-4.11 (SAS Institute Inc., Cary, NC).

Results

In Vitro *Motility Assays*

Actin filament sliding over myosins is shown in figure 1. Frequency histograms of velocities of actin filament sliding over myosins at baseline and with halothane or isoflurane (n = 150 in each group) are shown in figures 2 and 3, respectively. Motility velocities driven by myosin were not significantly different among baseline (2.70 ± 0.33 μm/s), 1 MAC halothane (2.72 ± 0.36 μm/s), and 2 MAC halothane (2.70 ± 0.40 μm/s). Similarly, motility velocities driven by myosin were not significantly different among baseline (2.73 ± 0.33 μm/s), 1 MAC isoflurane (2.72 ± 0.37 μm/s), and 2 MAC isoflurane (2.72 ± 0.40 μm/s). The effects of BDM on actin filament sliding are depicted in figure 4. Compared with baseline, the frequency histogram of actin-sliding velocities was significantly shifted toward lower values, and the mean velocity was nearly 50% slower after 2 mM BDM (1.44 ± 0.24 *vs.* 2.70 ± 0.30 μm/s; n = 150 filaments in each group; each $P < 0.001$).

ATPase Assays

Lineweaver–Burke plots of ATPase rate *versus* actin concentration in baseline conditions, 1 and 2 MAC halothane, and 1 and 2 MAC isoflurane are depicted in figure 5. Corresponding Vmax and Km values for actin are given in table 1. At any concentration tested, neither of the two anesthetics significantly modified Vmax and Km as compared with baseline. Effects of 2 mM BDM on

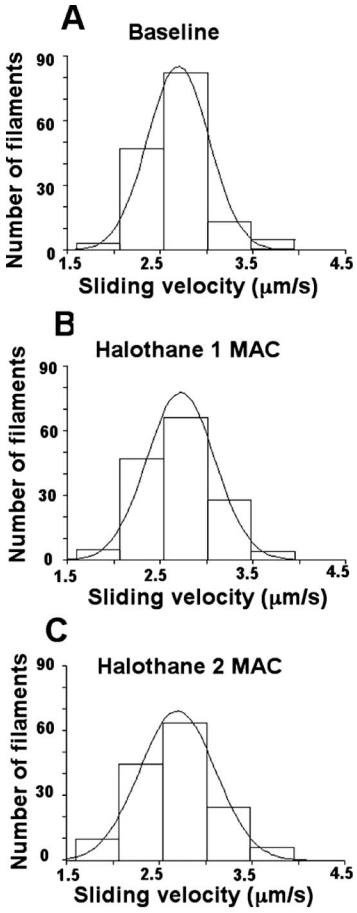

Fig. 2. Distribution of velocities of actin filament sliding over myosin at baseline (*A*), with 1 minimum alveolar concentration (MAC) halothane (*B*), and with 2 MAC halothane (*C*). Individual frame-to-frame velocities from tracked filaments are plotted in a frequency histogram. Each histogram contains data from 150 filaments taken from five separate assays. Temperature, 30°C.

enzymatic properties of myosin are depicted in table 1 and figure 5. Compared with baseline, BDM induced a nearly 56% reduction in Vmax but did not significantly affect Km.

Discussion

The current data show that clinically relevant concentrations of halogenated anesthetics did not interact with mechanical function or enzymatic properties of cardiac myosin, as assessed by nonsignificant changes in actin filament sliding, Vmax, and Km after halothane or isoflurane addition. Importantly, the results were not due to insensitivity of our preparation to drugs given that BDM, a well-known inhibitor of actomyosin function,[20,21] reduced both sliding velocity and Vmax.

Background

Although numerous studies have been performed with both skinned muscle and isolated myofibrillar preparations, the precise effects of halogenated anesthetics on

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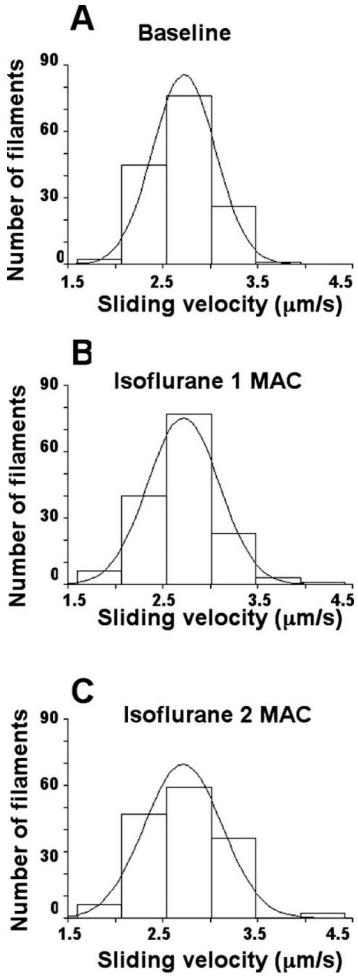

Fig. 3. Distribution of velocities of actin filament sliding over myosin at baseline (*A*), with 1 minimum alveolar concentration (MAC) isoflurane, (*B*) and with 2 MAC isoflurane (*C*). Individual frame-to-frame velocities from tracked filaments are plotted in a frequency histogram. Each histogram contains data from 150 filaments taken from five separate assays. Temperature, 30°C.

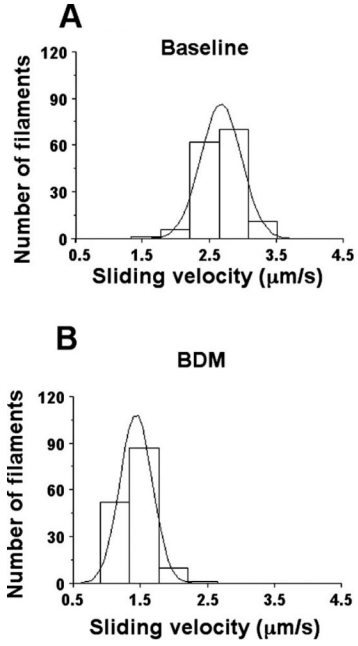

Fig. 4. Distribution of velocities of actin filament sliding over myosin at baseline (*A*) and with 2 mM 2,3-butanedione 2-monoxime (BDM) (*B*). Individual frame-to-frame velocities from tracked filaments are plotted in a frequency histogram. Each histogram contains data from 150 filaments taken from five separate assays. Temperature, 30°C. Additional information regarding this is available on the ANESTHESIOLOGY Web site at http://www.anesthesiology.org.

cardiac cross-bridge cycling have not been fully determined. Previous studies on cardiac skinned muscles suggested that halogenated anesthetics reduce the cross-bridge cycling rate,[8,12,31] in particular by reducing the cross-bridge attachment and detachment rates.[8,12] However, it is not easy to extrapolate skinned muscle experiments to intact myocardium because the skinning process can induce the loss of enzymes and second messengers, and this may affect both the $[Ca^{2+}]$–force relation and cross-bridge kinetics. Moreover, in these studies, cross-bridge kinetic parameters were not directly measured but estimated by using a simplified two-state model derived from the equations of Huxley.[32] At variance with these results, earlier studies investigating the dynamic stiffness of rabbit papillary muscles in Ba^{2+} contracture had suggested that volatile anesthetics did not alter actin–myosin ATPase kinetics but decreased the number of cross-bridge interactions, probably by decreasing the affinity of troponin C for Ba^{2+}.[33]

Halogenated Anesthetics and ATPase Activity

Assuming that one ATP molecule is hydrolyzed per cross-bridge cycle,[32] the ATPase activity of myosin directly reflects the overall duration of the cross-bridge cycle. Documented effects of halogenated anesthetics on myosin ATPase activity mainly concern studies performed on cardiac myofibrils. At high supratherapeutic concentrations, both halothane and isoflurane depress the actomyosin ATPase activity of myofibrils in different species, including rat,[13,15] dog,[14] and bovine,[16] although to varying extents. However, changes in enzymatic function depend on $[Ca^{2+}]$: Increasing $[Ca^{2+}]$ completely reverses the reduced ATPase activity induced by halothane,[14] and Ca^{2+}-activated myosin ATPase is increased after halothane.[17] Therefore, in all of these studies, indirect effects of halogenated anesthetics on cross-bridge could not be ruled out, these possible effects mainly being on regulatory proteins such as troponins, on calcium itself, or on both.

Halogenated Anesthetics and Cross-bridge Kinetics

In an attempt to overcome these difficulties, Langeron *et al.*[34] studied the effects of halogenated anesthetics in isolated diaphragmatic muscle, *i.e.*, a muscle in which halogenated anesthetics do not notably influence intracellular calcium movements. At therapeutic concentrations, halothane and isoflurane did not significantly modify the cross-bridge number, the elementary force per

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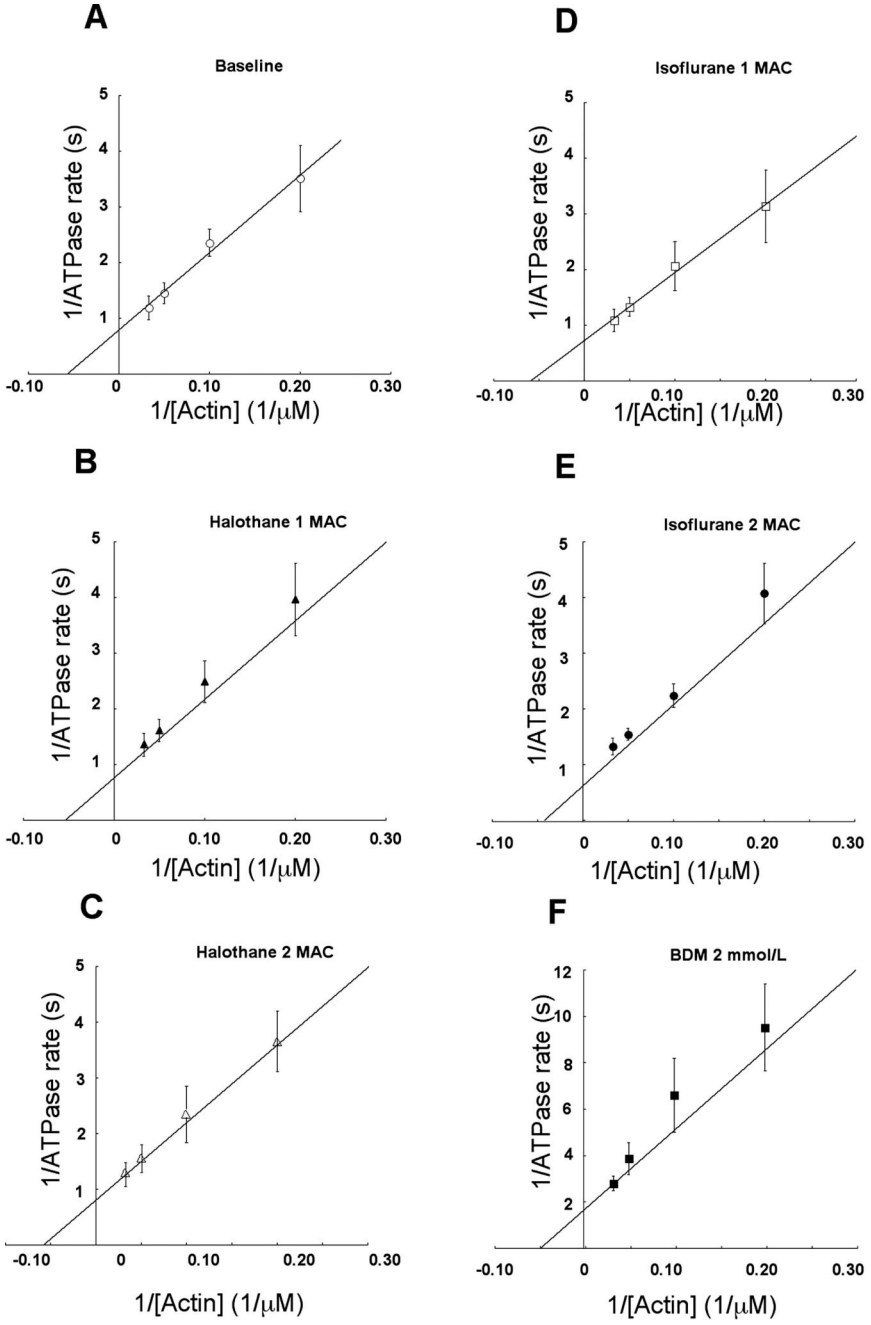

Fig. 5. Lineweaver–Burk double-reciprocal plots of cardiac myosin adenosine triphosphatase (ATPase) rates as a function of actin concentrations at baseline (*A*), with 1 minimum alveolar concentration (MAC) halothane (*B*), with 2 MAC halothane (*C*), with 1 MAC isoflurane (*D*), with 2 MAC isoflurane (*E*), and with 2,3-butanedione 2-monoxime (BDM, 2 mM) (*F*). Values are presented as mean ± SD of five individual measurements.

cross-bridge, the attachment and detachment constants, the duration of the cross-bridge cycle, or the mean cross-bridge velocity. However, rat diaphragm muscle strip differs considerably from cardiac muscle, and again the cross-bridge kinetic parameters were indirectly calculated from the equations of Huxley.[34]

Local Anesthetics and Myosin Function

To the best of our knowledge, our study is the first to directly characterize the effects of halogenated anesthetics on actin–myosin interactions at the molecular level using purified proteins. Only effects of local anesthetics on actomyosin motility have previously been investigated in such preparations.[19] It has been shown that local anesthetics such as lidocaine and tetracaine inhibit myosin motility in a dose- and pH-dependent manner, indicating the direct action of local anesthetics on the molecular motor. In contrast, local anesthetics do not affect binding of the actin filament to myosin, actomyosin ATPase activity, or the breaking force of the actomyosin complex,[16,19] thereby suggesting that these agents uncouple the mechanical event of myosin from its biochemical energy source. However, the finding that QX-314, a derivative of lidocaine, has no effect on myosin motility or enzymatic properties[16] strongly suggests that the effects of anesthetics on myosin function vary

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Table 1. Effects of Halothane and Isoflurane on Vmax and Km at Baseline, in the Presence of Halothane or Isoflurane, and after Addition of BDM

	Baseline	1 MAC Halothane	2 MAC Halothane	1 MAC Isoflurane	2 MAC Isoflurane	2 mM BDM
Vmax, s^{-1}	1.36 ± 0.11	1.20 ± 0.15	1.26 ± 0.15	1.37 ± 0.15	1.38 ± 0.15	0.60 ± 0.15*
Km, μmol	20 ± 3	19 ± 3	18 ± 5	17 ± 5	23 ± 5	26 ± 5

In each group, there were 5 separate experiments, except baseline, where there were 10 separate experiments. Values are presented as mean ± SD.

* $P < 0.001$ *vs.* baseline. No significant difference between control and volatile anesthetics groups.

BDM = 2,3-butanedione 2-monoxime; Km = association constant of myosin for actin; MAC = minimum alveolar concentration; Vmax = maximum actomyosin adenosine triphosphatase activity.

widely depending on the pharmacologic agent concerned. This may help to explain why at least some local anesthetics modulate cross-bridge function whereas the two halogenated anesthetics investigated in the current study do not.

Limitations

The experiments were performed at 30°C with myosin samples from healthy rats. Care must be taken before transferring results from *in vitro* studies on the effects of volatile anesthetics on motility assays and actin-activated ATPase activity to considerably more complex *in vivo* conditions. Nevertheless, such studies are indispensable for gaining insight into the physiologic cardiac pathways of halogenated anesthetics action and the pathologic consequences of muscular diseases.[25,26] We observed no significant depression of actomyosin ATPase activity *in vitro* with halothane or isoflurane at concentrations equivalent to 1 and 2 MAC. These results seem to be consistent with those previously obtained,[34] even if we do not know the precise anesthetic concentration that exists in the microenvironment of contractile proteins while using 1 and 2 MAC clinically in patients. Lastly, another explanation for the absence of depression of actomyosin activity with halogenated anesthetics in our study could be that the extraction procedure used might induce myosin motility and ATPase less sensitive to anesthetic depression.[14,35]

Conclusion

Our study showed that halothane and isoflurane at clinically relevant concentrations did not significantly affect *in vitro* myosin motility, the apparent binding affinity of myosin for actin or the maximum actin-activated ATPase activity. These results suggest that volatile anesthetics do not directly interact with cardiac myosin function and, therefore, that major cardiomyocyte sites for depression of contractility by halogenated anesthetics are to be found elsewhere.

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