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Effects of Isoflurane on Intracellular Calcium and Myocardial Crossbridge Kinetics in Tetanized Papillary Muscles

James D. Hannon, M.D., Mark J. Cody, B.A.,† Philippe R. Housmans, M.D, Ph.D.‡*

Background: **Isoflurane depresses the intracellular Ca^{2+} transient and force development during a twitch, but its effects on crossbridge cycling rates are difficult to predict because of the transient nature of the twitch. Measurements of the effects of isoflurane on crossbridge cycling kinetics during tetanic contractions, which provide a steady state level of activation in intact cardiac muscle, have not been previously reported.**

Methods: **Ferret right ventricular papillary muscles were isolated, and superficial cells were microinjected with the bioluminescent photoprotein aequorin to monitor the intracellular Ca^{2+} concentration. The rate of tension redevelopment (k_{TR}) was measured during steady state isometric activation (tetanic stimulation, frequency 20 Hz, 1 μM ryanodine, temperature = 30°C) in the absence of isoflurane (2, 6, and 12 mM extracellular [Ca^{2+}]) and in the presence of 0.5, 1.0, and 1.5 minimum alveolar concentration isoflurane (12 mM extracellular [Ca^{2+}]).**

Results: **Intracellular [Ca^{2+}], isometric force, and k_{TR} all increased when the extracellular [Ca^{2+}] increased. Isoflurane (0.5, 1.0, and 1.5 minimum alveolar concentration) caused intracellular [Ca^{2+}], isometric force, and k_{TR} to decrease in a dose-dependent manner in the presence of 12 mM extracellular [Ca^{2+}]. In the presence of increasing concentrations of isoflurane, the relation between intracellular [Ca^{2+}] and force remained unchanged, whereas the relation between intracellular [Ca^{2+}] and k_{TR} was shifted toward higher [Ca^{2+}].**

Conclusions: **These results indicate that isoflurane depresses myocardial crossbridge cycling rates. It appears that this effect is partially mediated by a decrease in the intracellular [Ca^{2+}]. However, additional mechanisms must be considered to explain the shift of the relation between intracellular [Ca^{2+}] and k_{TR} toward higher [Ca^{2+}].**

MYOCARDIAL contraction results from a sequence of events that includes depolarization of the plasma membrane, release and sequestration of Ca^{2+} by the sarcoplasmic reticulum (Ca^{2+} transient), Ca^{2+} binding to troponin C, and crossbridge cycling. Volatile anesthetics depress the contractility of isolated myocardium in a dose-dependent reversible manner.[1–4] However, mainly because of the large number of potential sites of interaction, the mechanisms by which they exert this effect are still under active investigation.[5,6]

Inotropic mechanisms can be classified according to their ability to alter (1) the intracellular Ca^{2+} transient ("upstream" mechanisms), (2) the affinity of troponin C for Ca^{2+} ("central" mechanisms), and (3) the response of the myofibrillar proteins to a given level of Ca^{2+} ("downstream" mechanisms).[7] Recent studies have exposed the ability of the volatile agents to depress contractility by upstream mechanisms. They are known to decrease the intracellular Ca^{2+} transient[8–10] and have been shown to affect Ca^{2+} influx through surface membrane Ca^{2+} channels and intracellular Ca^{2+} release and sequestration by the sarcoplasmic reticulum.[11]

There is also evidence that the volatile anesthetics act by altering calcium sensitivity (a central mechanism). It has been reported that halothane, enflurane, and isoflurane all appear to reduce myocardial Ca^{2+} sensitivity at greater than 0.5 minimum alveolar concentration (MAC) in intact muscle.[10,12] At 1 MAC, the reduction of myocardial calcium sensitivity was most pronounced in the presence of isoflurane and was estimated to play only a minor role in the negative inotropy of the volatile anesthetics. Similarly, Bosnjak *et al.*[8] reported that isoflurane, but not halothane or enflurane, reduced the Ca^{2+} sensitivity of intact cardiac muscle.

Little information is available about anesthetic effects on crossbridge cycling kinetics in the heart. Studies of anesthetic effects in myocardium with a porous surface membrane (skinned muscle) suggest that at high concentrations, volatile agents can affect crossbridge cycling.[13–15] Extrapolation of these findings to intact myocardium is difficult because the skinning process can result in the loss of second messengers and enzymes that can affect the [Ca^{2+}]–force relation and could alter crossbridge kinetics as well.[16,17]

Therefore, we determined the effect of isoflurane on crossbridge kinetics in intact papillary muscles by measuring the rate of tension redevelopment (k_{TR}) during the plateau phase of a tetanic contraction, when force and intracellular [Ca^{2+}] have reached a steady state.[16,17]

Methods

Tissue Preparation

All experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Mayo Foundation. Protocols were completed in accordance with National Institutes of Health guidelines and in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council). Adult male ferrets (1.2 kg, Marshall Farms,

* Assistant Professor, † Research Technologist, ‡ Professor.

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Address reprint requests to Dr. Hannon, Department of Anesthesiology, Mayo Foundation, Rochester, Minnesota 55905. Address electronic mail to: hannon.james@mayo.edu.

North Rose, NY) were anesthetized with isoflurane in 100% oxygen, and the hearts were rapidly excised and placed in oxygenated physiologic salt solution. The aorta was then cannulated within 1 min, and the hearts were perfused with oxygenated physiologic salt solution. An appropriate papillary muscle was selected (cross-sectional area = 0.69 ± 0.20 mm^2, length = 5.1 ± 1.0 mm, n = 8) and excised from the right ventricle.

Detection of the Intracellular Ca^{2+} Transient

Aequorin (Friday Harbor Laboratories, Friday Harbor, WA) was introduced into superficial cells of the papillary muscles with a glass micropipette (1.5-mm OD, thin walled; World Precision Instruments, Sarasota, FL).[18] The light emitted by aequorin was detected with a photomultiplier (9235QA; Electron Tubes Inc., Rockaway, NJ) and a current-to-voltage converter (Melles Griot, Irvine, CA). Digital signal averaging was used to obtain a satisfactory signal-to-noise ratio. Light measurements were converted into units of fractional luminescence (L/L_{max}) by dividing them by the peak light intensity (L_{max}) recorded during the optical conditions of the experiment when all of the remaining aequorin was exposed to a saturating concentration of calcium.[19] To obtain L_{max}, the cell membranes of the preparation were lysed with 4% Triton X-100 (Sigma, St. Louis, MO), and the light emitted was integrated electronically.[18] L_{max} was corrected for consumption of aequorin by subtracting the amount of light emitted during each tetanus.[16] The amount of aequorin consumed during the course of an entire experiment averaged less than 2% of the total injected (mean = 1.4 ± 1.1%). The intracellular [Ca^{2+}] was determined from the fractional luminescence by referring to an *in vitro* calibration curve.[18,19] The entire protocol was conducted on seven papillary muscle preparations and all but the measurements at 1.5 MAC on one other.

Experimental Setup for Force and Force Redevelopment Measurements

Muscles were mounted in a chamber that contained 70 ml of physiologic salt solution maintained at a temperature of 30°C. The muscle was clamped at its lower end to a small plastic block. Electrical stimulation was accomplished with square-wave unipolar pulses of 5-ms duration delivered through platinum field electrodes at 20% above threshold intensity. To reduce the compliance of the preparation, the chorda tendinea was lashed to a thin glass strand (100-μm diameter, fiber optic) attached to the arm of a servo-controlled electromagnetic muscle lever operated in the isometric mode (Model 300B; Cambridge Technology, Inc., Cambridge, MA).

Muscle length was adjusted to a point at which the tension developed was optimal. The diameter and length of the muscle from the clamp to the chorda tendinea were measured *in situ*. The bathing solution was continuously bubbled with 100% oxygen, buffered with 3-[*N*-morpholino] propanesulfonic acid (MOPS) at pH 7.4, and contained 140 mM Na^+, 5 mM K^+, 2.0 mM Ca^{2+}, 1 mM Mg^{2+}, 103.5 mM Cl^-, 1 mM SO_4^{2-}, 5 mM MOPS, 10 mM glucose, and 20 mM acetate.

Appropriate conditions for tetanic contractions were produced by exposing the muscles to1 μM ryanodine (Calbiochem, San Diego, CA).[20] Muscles were allowed to equilibrate for 30 min after exposure to ryanodine while being stimulated at 0.25 Hz. Fused tetani were produced with 20 Hz stimulation for 6 s. Tetani were induced at 2-min intervals. To achieve low, intermediate, and high levels of activation, the muscles were exposed to 2, 6, and 12 mM extracellular [Ca^{2+}].

The constant for k_{TR} was determined with a length-change protocol that results in the dissociation of crossbridges. In this protocol, muscle length is quickly (< 1 ms) reduced by 20% to allow the muscle to shorten for a brief period (50 ms) and then rapidly returned to the initial length. Crossbridges then reattach, and tension redevelops at a rate determined by a least squares fit to a single exponential curve [$T = T_0 (1 - e^{-kt})$]. A computer program written in graphical software language (LabView, National Instruments, Austin, TX) was used to control the experiments. Signals were recorded by sampling at 1,000 Hz and digitizing with a 12-bit data acquisition board (AT-MIO16-L9, National Instruments).

Administration of Anesthetic

In all experiments, an inline calibrated anesthetic vaporizer was used to add the appropriate concentration of isoflurane to the preparation. Concentrations of volatile anesthetics in the gas over the bathing solution were monitored by Raman spectroscopy (Ohmeda Rascal II; Ohmeda, Madison, WI) and were also verified in the bathing solution by gas chromatography (5880A; Hewlett-Packard, Palo Alto, CA).[21] The 1-MAC value for isoflurane (Abbott Laboratories, North Chicago, IL) in the ferret at 30°C (1.16%) was calculated from the 1-MAC value at 37°C (1.5% gas phase)[22] because the MAC values at different temperatures are essentially constant when expressed as aqueous phase concentrations.[23]

Experimental Protocols

A simple two-state model of crossbridge cycling was used to interpret the mechanical data obtained in these experiments.[24–27] In this model, crossbridges exist in two states: non–force-generating (A), and force-generating (B). The transitions between the two states are described by two apparent rate constants, one for the transition to the force-generating state (f_{app}), and one for the transition to the non–force–generating state (g_{app}). This model treats crossbridge formation as a reversible first-order chemical reaction. In theory, the total number of active crossbridges per half sarcomere and g_{app} are fixed, and f_{app} increases during activation.[27]

During steady state conditions, as in a tetanus, the rate of formation of crossbridges is equal to the rate of dissociation of crossbridges and the equilibrium between force-generating (B) and non–force-generating (A) crossbridges is described by:

$$f_{app}[A] = g_{app}[B] \quad (1)$$

During these circumstances, if all crossbridges start in the non–force-producing state, then the rate of k_{TR} will reflect the sum of the forward and reverse rate constants:

$$k_{TR} = f_{app} + g_{app} \quad (2)$$

In addition, according to this model, the force is proportional to the ratio:

$$C \cdot f_{app}/(f_{app} + g_{app}) \quad (3)$$

where C is a constant that describes the total number of active crossbridges and the average force per crossbridge. Therefore, the derived relation between k_{TR} and normalized force derived from the two-state model is:

$$k_{TR} = g_{app}/(1 - F_n + (F_n \cdot g_{app})/k_{TRmax}) \quad (4)$$

where F_n is the normalized force and k_{TRmax} is the maximum value of k_{TR}.

Statistical Analysis

Results are reported as the mean ± SD. Force was normalized for muscle cross-sectional area. The length and diameter were measured at L_{max}, and the muscle was assumed to approximate a cylinder. Linear or nonlinear regression was used to fit the data. Statistical significance ($P < 0.05$) was determined by a paired *t* test or one-way repeated-measures analysis of variance with the Dunnett test for multiple comparisons *versus* control.

Results

Figure 1 shows a representative recording of muscle length and tension during a tetanic contraction in a right ventricular papillary muscle. A high level of steady force has been achieved before decreasing the muscle length by 20%. When the muscle length falls, the tension rapidly decreases to zero and the muscle starts to shorten. The muscle length is then reextended to L_{max}, breaking any crossbridges that may have formed during shortening. The tension therefore starts out at near the resting level and quickly redevelops as crossbridges reform.

We found that intracellular $[Ca^{2+}]$, isometric force, and k_{TR} all increased as the extracellular $[Ca^{2+}]$ was increased (table 1). Figure 2 shows a recording of aequorin luminescence and isometric force at 2, 6, and 12 mM extracellular $[Ca^{2+}]$. There was a progressive increase in the steady level of intracellular $[Ca^{2+}]$ as the extracellular $[Ca^{2+}]$ was elevated. Superimposable records could be obtained from successive tetani so that several consecutive tetani could be averaged to improve the signal-to-noise ratio.[16] After a suitable number of tetani (4–7) had been recorded for intracellular $[Ca^{2+}]$ measurement, the k_{TR} protocol was imposed during the subsequent tetanus. Although the light-gathering apparatus used in these experiments is designed to minimize movement artifact, we did not attempt to measure intra-

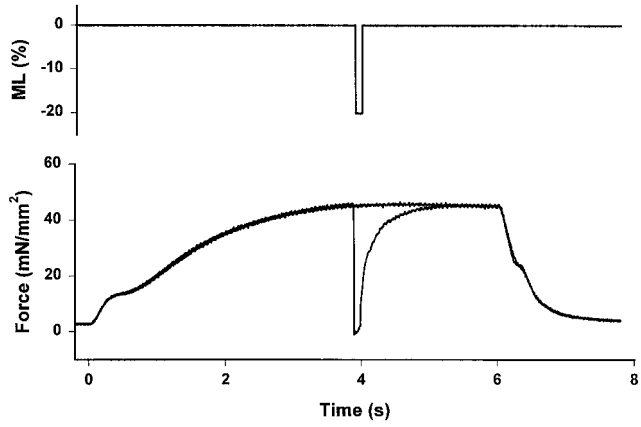

Fig. 1. Simultaneous recording of force and muscle length (ML) during maximal steady activation (six second tetanus, 6 mM extracellular $[Ca^{2+}]$) in a ferret right ventricular papillary muscle (temperature = 30°C). The upper trace shows the rapid release of muscle fiber length followed by a reextension to the original length (L_{max}). The lower trace shows the time course of two tetanic contractions superimposed. One was recorded in the presence of the length step and the other in its absence.

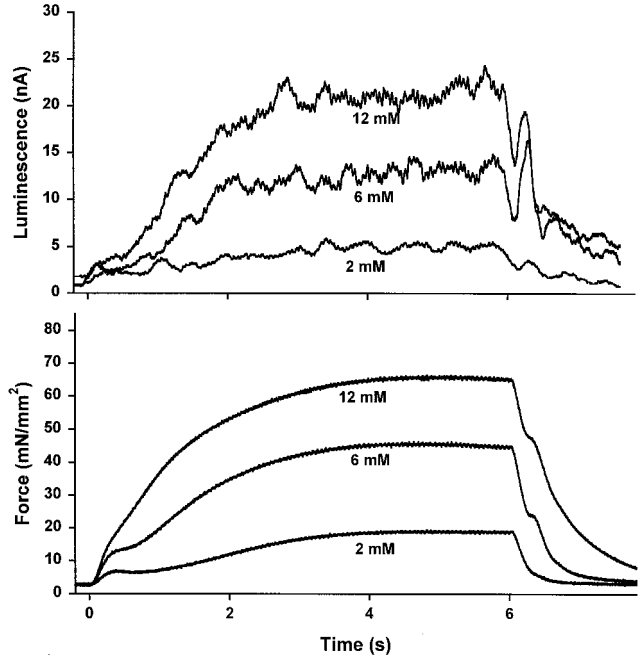

Fig. 2. Recordings of aequorin light signals (*top*) and isometric force (*bottom*) during tetani at 2, 6, and 12 mM extracellular $[Ca^{2+}]$. The level of activation and the intracellular $[Ca^{2+}]$ increased with increasing extracellular $[Ca^{2+}]$. Average of four tetani.

Table 1. Isometric Force, $[Ca^{2+}]_i$, and k_{TR} in Isolated Papillary Muscles

Isoflurane (% atm)	$[Ca^{2+}]_o$ (mM)	$[Ca^{2+}]_i$ (nM)	Force (mN/mm^2)	k_{TR} (s^{-1})
0	2	410 ± 77*	24 ± 15*	2.2 ± 0.4*
0	6	601 ± 195*	53 ± 23*	4.3 ± 1.4*
0	12	751 ± 212	67 ± 21	6.0 ± 2.2
0.5	12	669 ± 249	63 ± 20	4.6 ± 1.7*
1.0	12	638 ± 208*	55 ± 7*	3.5 ± 1.3*
1.5	12	613 ± 198*	44 ± 23*	2.4 ± 0.9*

Mean ± SD. n = 8 except n = 7 at 1.5 MAC.

* $P < 0.05$ compared with 12 mM $[Ca^{2+}]_o$, 0% isoflurane.

$[Ca^{2+}]_i$ = intracellular calcium concentration; k_{TR} = rate constant of tension redevelopment; $[Ca^{2+}]_o$ = extracellular calcium concentration; force = active tetanic force.

cellular $[Ca^{2+}]$ during the length change to eliminate this possibility.

Isoflurane caused k_{TR} to decrease when the muscles were tetanized in 12 mM extracellular $[Ca^{2+}]$ (table 1). Figure 3 shows the effect of extracellular $[Ca^{2+}]$ and isoflurane on k_{TR}. k_{TR} was clearly faster when the extracellular $[Ca^{2+}]$ was increased from 2 to 12 mM. The k_{TR} was 2.2 s^{-1} in 2 mM extracellular $[Ca^{2+}]$ and increased to 6.9 s^{-1} when the extracellular $[Ca^{2+}]$ was increased to 12 mM. In this example, 1 MAC isoflurane slowed k_{TR} to 3.5 s^{-1} (in the continuing presence of 12 mM extracellular $[Ca^{2+}]$).

Isoflurane caused the intracellular $[Ca^{2+}]$ to decrease in a concentration-dependent manner in the presence of 12 mM extracellular $[Ca^{2+}]$ (table 1). Figure 4 shows the effect of 0.5, 1.0, and 1.5 MAC isoflurane on k_{TR} and intracellular $[Ca^{2+}]$. The relation between intracellular $[Ca^{2+}]$ and k_{TR} in the absence of anesthetic (obtained by changing the extracellular $[Ca^{2+}]$) is also shown. The figure shows that the relation between intracellular

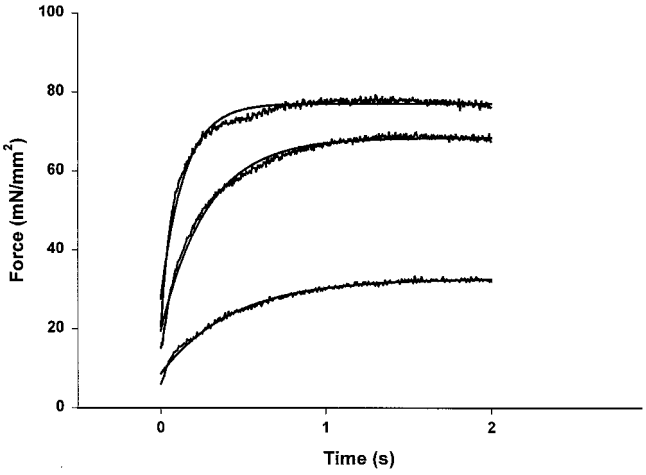

Fig. 3. Force redevelopment recorded during tetanic stimulation. For clarity, the time at which the trace starts has been adjusted to the end of the length step. The bottom trace was obtained in 2 mM extracellular $[Ca^{2+}]$. The upper trace was obtained in 12 mM extracellular $[Ca^{2+}]$. The middle trace was obtained in 12 mM extracellular $[Ca^{2+}]$ plus 1 MAC isoflurane.

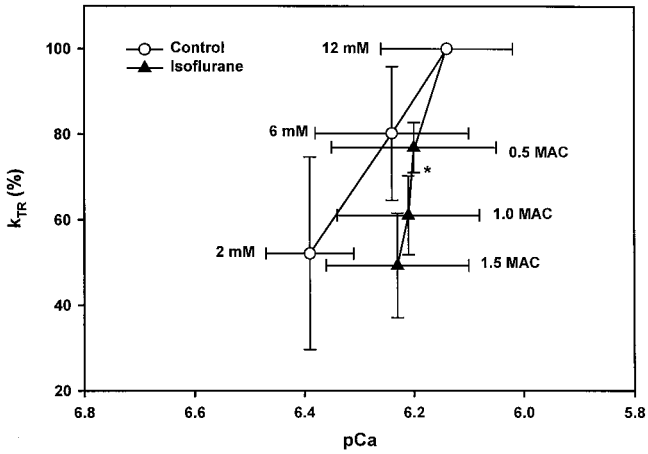

Fig. 4. The effect of increasing isoflurane concentration on the relation between intracellular $[Ca^{2+}]$ and force redevelopment. Tension redevelopment (k_{TR}) was normalized to the value obtained in 12 mM extracellular $[Ca^{2+}]$. pCa = $-\log[Ca^{2+}]$. n = 8 for each concentration, except n= 7 for 1.5 MAC. *$P < 0.05$ for mean slope of fitted lines in the presence of isoflurane compared with control conditions.

$[Ca^{2+}]$ and k_{TR} is shifted toward higher $[Ca^{2+}]$ in the presence of isoflurane. On the other hand, increasing isoflurane from 0.5 to 1.5 MAC did not appear to alter the relation between intracellular $[Ca^{2+}]$ and force in the presence of 12 mM extracellular $[Ca^{2+}]$, as shown in figure 5.

The relation between isometric force and k_{TR} is altered in the presence of isoflurane (figure 6). The data points obtained in the presence of anesthetic fall below the curve obtained in its absence. During these circumstances, g_{app} is decreased in the presence of anesthetic from 2.7 ± 0.8 to 1.7 ± 0.9 s^{-1} ($P < 0.05$).

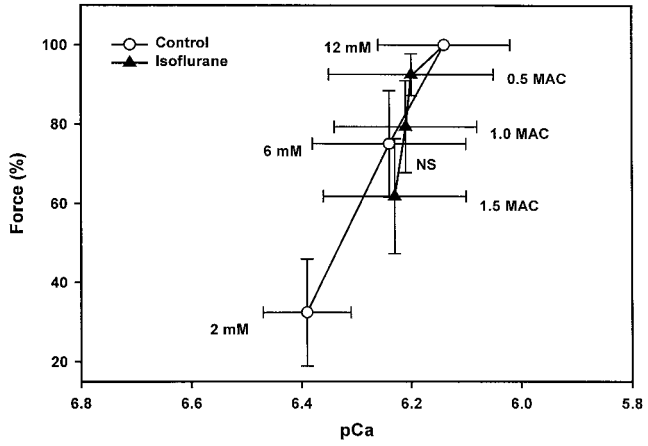

Fig. 5. The effect of increasing isoflurane concentration on the relation between intracellular $[Ca^{2+}]$ and force. Tetanic force was normalized to the value obtained in 12 mM extracellular $[Ca^{2+}]$. n = 8 for each concentration. NS = $P > 0.05$ for mean slope of fitted lines in the presence of isoflurane compared with control conditions.

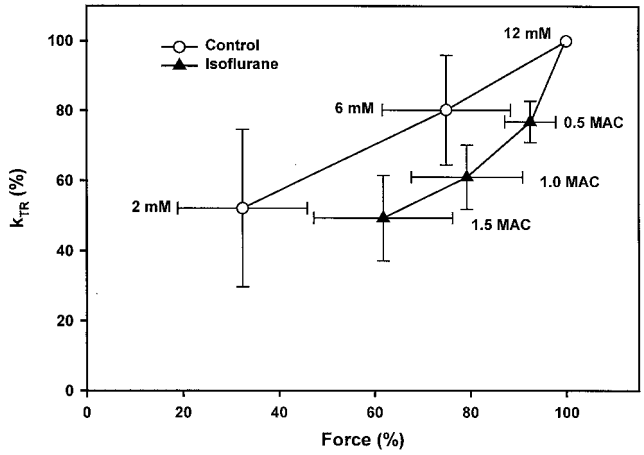

Fig. 6. The relation between k_{TR} and isometric force in the presence of increasing extracellular calcium concentration (control) and in the presence of increasing isoflurane concentration (isoflurane).

Discussion

We determined the effect of isoflurane on crossbridge kinetics in intact papillary muscles by measuring the rate of k_{TR} during the plateau phase of a tetanic contraction, when force and intracellular [Ca^{2+}] have reached a steady state.[16,17] The results of these experiments indicate that isoflurane slows myocardial crossbridge kinetics in the heart. This is the first report of such an effect on crossbridge cycling (k_{TR}) in intact myocardium.

Previous studies have focused on mechanical measurements in skinned cardiac muscle at room temperature or lower.[13,14] Skinned preparations are useful because they have their surface membranes disrupted, rendering them permeable to small ions and molecules. In this way, the ionic composition of the solution bathing the myofibrils can be precisely controlled and the calcium concentration altered to produce varying levels of activation. Thus, a clearer picture of the effects of an intervention directly on the function of the myofibrillar apparatus can be obtained.

On the other hand, soluble second messengers, enzymes, and perhaps other unknown small molecules would be expected to be lost in the skinning process, and this fact might alter the conclusions of such studies.[28] The skinning process clearly alters Ca^{2+} sensitivity and the steepness of the [Ca^{2+}]–force relation.[16,17] The effects of interventions on processes such as phosphorylation and dephosphorylation would be lost unless the proper enzymes were included. Therefore, it is very important to examine the effects of the halogenated volatile anesthetics on crossbridge kinetics in intact myocardium.

The link between [Ca^{2+}] and k_{TR} has been well documented in skinned skeletal muscle, where k_{TR} is known to increase exponentially as the level of activation by calcium increases.[27,29] The effect of Ca^{2+} on k_{TR} in intact and skinned cardiac muscle is slightly more controversial. Hancock *et al.*[30,31] reported that k_{TR} in intact ferret cardiac muscle was unaffected by altering the extracellular or intracellular [Ca^{2+}], but they used a different protocol to control the muscle length. In their protocol, muscle length was decreased by 3% but was not returned to L_{max}. Other investigators have found that calcium increases k_{TR} in skinned rat trabeculae,[32] skinned rat myocytes,[33] and, most recently, in intact rat trabeculae.[34] Our results, obtained using a similar length-change protocol, indicate that k_{TR} in intact cardiac muscle varies with the level of activation as achieved by altering the level of extracellular [Ca^{2+}] (fig. 4).

The length-step protocol used in these experiments is essentially the same as that used by other investigators in skinned muscle.[25–27,29,32] The results obtained in this investigation are not influenced by length-dependent mechanisms such as Starling's law because the muscle was returned to L_{max} before the measurement started. In a similar manner, it is unlikely that the length-change protocol altered the function of the surface membrane or the sarcoplasmic reticulum.

Volatile anesthetics are known to alter the intracellular [Ca^{2+}] during a twitch.[8–10,35] For isoflurane, this effect appears to be primarily caused by an inhibition of Ca^{2+} entry through surface membrane Ca^{2+} channels. Therefore, it seemed likely to us that isoflurane would slow crossbridge transitions in normal muscle with an intact surface membrane primarily by decreasing the intracellular [Ca^{2+}]. However, if isoflurane decreased the rate of crossbridge cycling only by this mechanism, then we would expect to find that the relation between intracellular [Ca^{2+}] and k_{TR} would not be altered in the presence of anesthetic. On the contrary, we found that isoflurane shifts the relation between intracellular [Ca^{2+}] and k_{TR} toward higher [Ca^{2+}] (fig. 4). This indicates that isoflurane may influence crossbridge cycling by another mechanism.

The observation that k_{TR} was changed to a greater extent than isometric force in the presence of isoflurane (fig. 5) is consistent with this interpretation and suggests that both f_{app} and g_{app} probably decrease in the presence of isoflurane. The crossbridge model can illustrate this concept. According to the model, k_{TR} is the sum of f_{app} and g_{app} (equation 2), and force is proportional to the ratio of f_{app} and k_{TR} (equation 3). If only f_{app} decreases with decreasing activation, then the points obtained in the presence of isoflurane should fall along the curve obtained by changing the extracellular [Ca^{2+}]. However, if only g_{app} decreased in the presence of isoflurane, the force would be expected to increase, since each crossbridge would be expected to remain attached for a longer time.

Surprisingly, isoflurane did not appear to alter the relation between intracellular [Ca^{2+}] and isometric force during the conditions of the experiment. One might

expect to see a change in calcium sensitivity because it has been reported that crossbridge attachment can influence calcium binding to troponin C. However, the conditions used in these experiments are different from those used by other investigators to show that isoflurane decreases calcium sensitivity.[8,12]

In summary, these results show that isoflurane alters myocardial crossbridge kinetics in intact heart muscle. The depression of crossbridge cycling seems to be caused, in part, by an indirect effect of the volatile anesthetics on intracellular Ca^{2+}, but a more direct effect at the level of the crossbridge is the most likely explanation for the decrease in crossbridge detachment rate (g_{app}).

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