Anesthesiology
70:837–842, 1989

Isoflurane and Halothane Inhibit Tetanic Contractions In Rabbit Myocardium In Vitro

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Rabbit right ventricular papillary muscles were tetanized by rapid stimulation in the presence of 1 μM ryanodine, an inhibitor of sarcoplasmic reticular function. Tetanic contractions elicited in this manner increased in strength as extracellular calcium concentration $[Ca^{+2}]_{ext}$ was raised from 0.5 to 5 mM, exhibited saturation behavior above $[Ca^{+2}]_{ext}$ = 5 mM, and were blocked by nifedipine. Accompanying membrane potentials depolarized to +10 mV and repolarized to −60 mV between stimuli. These data suggest that rabbit myocardial tetany is supported in large part by extracellular calcium influx *via* slow (L-type) calcium channels, consistent with similar recent findings in the ferret. At $[Ca^{+2}]_{ext}$ = 2.5 mM, isoflurane (0.6–2.3%, gas phase) and halothane (0.4–1.5%) inhibited the strength of tetanic contractions in dose-dependent fashion. At $[Ca^{+2}]_{ext}$ = 20 mM neither isoflurane (1.2%) nor halothane (0.8%) inhibited tetanic contraction strength. These data demonstrate that isoflurane and halothane inhibit contractile activity that is dependent on trans-sarcolemmal calcium influx *via* pathways independent of the ryanodine-sensitive sarcoplasmic reticulum. The exact sites of inhibition (*e.g.*, slow channel *vs.* intracellular transit *vs.* myofibrillar binding) are not identified, but inhibition by either anesthetic may be competitively reversed by high extracellular calcium concentrations. (Key words: Anesthetics, volatile: halothane, isoflurane. Heart: action potential; myocardial contractility; negative inotropy; sarcolemmal calcium influx; tetanic contraction.)

THE NEGATIVE INOTROPIC effect of volatile anesthetics is believed to arise in large part through alteration in the availability of contractile activation calcium that reaches the myofibrils during systole.[1]

The transient rise in intracellular free calcium induced by the excitation-contraction coupling mechanism is derived from functionally distinguishable pools:[2] calcium entering from outside the cell (free extracellular calcium and calcium previously bound to superficial sarcolemmal sites) and calcium released from sarcoplasmic reticular stores within the cell (having been stored subsequent to previous contractions). Using techniques that highlight one or the other of these calcium sources, we[3–6] and others[7–11] have demonstrated that halothane is inhibitory both to extracellular calcium entry and to sarcoplasmic reticular calcium release.

Controversy exists, however, in ascribing the major site of contractile inhibition by isoflurane. Work in our laboratory using rabbit papillary muscle has shown isoflurane to be a potent inhibitor of low-frequency steady-state contractions,[5] which predominantly depend upon extracellular calcium influx. Moreover, we have demonstrated that isoflurane has no effect on potentiated-state contractile strength,[6] which depends predominantly on sarcoplasmic reticular calcium. These findings support the postulate that the sarcolemma (or events involved in extracellular calcium entry) is the major site of negative inotropic action of isoflurane. In apparent contrast, Lynch[12] has reported that the late-peaking contractions of partially depolarized (26 mM external K^+) guinea pig papillary muscle are inhibited to 23% of control by 4% isoflurane, a dose which only depresses the rate of upstroke of the associated slow action potentials (measure of Ca^{+2} mediated slow inward current) to 83% of control. This observation suggests that inhibition of extracellular calcium entry contributes little to isoflurane's inhibition of contractility.

Recently, Yue *et al.*[13] and Marban *et al.*[14] have described tetanization of ferret papillary muscle as a technique for producing steady contraction totally dependent on the influx of extracellular calcium for contractile activation. When muscles were stimulated at high frequency after exposure to ryanodine (an inhibitor of sarcoplasmic reticular function[15]), steady contractile tension was achieved that varied proportionately to external calcium from 0.5 to 5 mM, saturated above 10 mM (corresponding to $[Ca^{+2}]_i$ = 0.85 μM by aequorin luminesence measurement), was blocked by nitrendipine and nifedipine, and was enhanced by Bay K 8644 (a dihydropyridine calcium channel agonist).

The present work was undertaken first to ascertain appropriate conditions under which tetanic contractions could be established in rabbit papillary muscle, the preparation in which this laboratory has previously studied anesthetic-induced negative inotropy. Second, we sought to establish what effect, if any, isoflurane and halothane

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Received from the Department of Anesthesiology, Rochester General Hospital, Rochester, New York; and the Department of Anesthesiology, University of Wisconsin Medical School, Madison, Wisconsin. Accepted for publication January 5, 1989. Supported in part by National Institutes of Health Research Grant GM-29527 and by a grant from the University of Wisconsin Department of Anesthesiology Research and Development Fund. Presented in part at the annual meeting of the American Society of Anesthesiologists, Atlanta, Georgia, October, 1987.

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would have on contractions totally dependent on extracellular calcium entry in a preparation rendered functionally devoid of sarcoplasmic reticulum by treatment with ryanodine.

Materials and Methods

All studies were approved by the University of Wisconsin Research Animal Care and Use Committee. New Zealand white rabbits weighing 2.1 ± 0.2 kg (mean ± S.D.) were anesthetized by intravenous injection of pentobarbital (approximately 45 mg/kg). The heart was rapidly excised and bathed in oxygenated Krebs-Henseleit buffer containing 2.5 mM calcium (vide infra). Right ventricular papillary muscles (0.91 ± 0.18 mm diameter by 4.6 ± 1.2 mm length) were dissected free and mounted in a 10-ml tissue superfusion chamber maintained at 30° C. This temperature was chosen to be consistent both with our earlier studies,[3-6] and with Yue *et al.* and Marban *et al.*'s tetany experiments.[13,14] The base of each muscle was tied with 5-0 silk to a stationary support and the tendinous end was similarly tied to a connecting rod from a Gould-Statham UTC-2 force transducer. The diameter of each muscle was measured under 1.0 gm load with an optical micrometer (assuming a cylindrical shape), and resting tension was readjusted to 1.0 gm per mm^2 (near the peak in the length-tension relationship). Muscles were field stimulated using platinum electrodes driven by a Grass® S48 laboratory stimulator. Force of contraction was recorded with a Gilson® polygraph. All muscles were equilibrated for 1 h with 0.1 Hz stimulation at 1.5 times threshold voltage before performing tetany experiments. In several muscles, intracellular potential was monitored by impaling the tissue with a 3 molar KCl-filled glass microelectrode referenced against a Ag/AgCl bath electrode. These potentials were amplified with a Dagen® model 8500 preamplifier and recorded on a Norland® model 2001 programmable digital oscilloscope. The buffer used at physiologic (2.5 mM) and lower calcium concentrations was the same as in our previous studies, a modified Krebs-Henseleit bicarbonate containing: NaCl, 115 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; NaH_2PO_4, 1.2 mM; Na_2SO_4, 1.2 mM; $NaHCO_3$, 25 mM; glucose, 5.6 mM; ethylenediaminetetraacetate, 50 μM (added to chelate trace metals); and equilibrated to *p*H 7.4 by constant bubbling with a 95% O_2 + 5% CO_2 gas mixture. Experiments at higher calcium concentrations could not be performed in this buffer due to precipitation of calcium phosphate salts. For these, a *p*H 7.4 HEPES-acetate buffer was used containing: NaCl, 108 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), 5 mM; sodium acetate, 20 mM; glucose 5.6 mM; ethylenediaminetetraacetate, 50 μM; and equilibrated with 100% O_2. In all experiments, $CaCl_2$ was added to achieve the desired extracellular calcium concentration.

A preliminary series of experiments was undertaken to define optimum conditions for producing tetanic contractions in the rabbit papillary muscle preparation. In these experiments, ryanodine concentration, stimulation frequency, stimulation voltage, and rest interval were varied. As in the ferret,[13,14] a ryanodine concentration of 1 μM proved adequate, with similar results achieved at 2, 3, or 5 μM. All muscles were equilibrated for 1 h after removal and for an additional hour after the addition of ryanodine to the superfusion buffer. Optimum stimulation occurred at twice threshold voltage using 40 msec rectangular wave voltage pulses at 4 Hz. At less than 4 Hz, diastolic tension was not maximal, and at more rapid stimulus rates the contractile response frequency fell abruptly to half the stimulus rate. Tetani were found to be quite reproducible when sustained for 4 s and separated by "rest" intervals of 56 s during which twitches were evoked at 0.4 Hz. Tetanic contraction strength was defined as the maximum diastolic tension achieved during 4 Hz stimulation.

The effect of the slow calcium channel antagonist nifedipine on tetanic contraction strength was tested in a series of four muscles. Using 2.5 mM extracellular calcium concentration, tetanic contraction strength was measured before and 1 h after the addition of 1 μM nifedipine (with room lights dimmed during the incubation period). Results were compared with a paired *t* test statistic defining significance as $P < 0.05$. The effect of external calcium concentration on tetanic contraction strength was tested in a series of experiments (N = 4) in which calcium concentration was varied from 0.5 to 20 mM. An equilibration period of 30 min followed each calcium concentration change.

Anesthetics were introduced *via* the superfusion gas with calibrated Drager® vaporizers and steady-state liquid phase concentrations were achieved within 15 min as assayed by extraction and gas chromatography. The effect of isoflurane on tetanic contractions at 2.5 mM external calcium concentration was assessed in eight muscles. Isoflurane 0.0, 0.6, 1.2, 1.7, or 2.3 volume percent (gas phase) was introduced in random sequence with equilibration for 30 min prior to the measurement of tetanic contraction strength. Similarly, in a series of five muscles, halothane 0.4, 0.8, 1.1, and 1.5 volume percent was tested.

The effect of anesthetics at saturating levels of external calcium was assessed in four muscles. In each of these, stable tetani were established with 20 mM calcium in the superfusion buffer, then the effect of 1.2% isoflurane and 0.8% halothane (introduced in random order with a 30-min washout period between) was measured.

Anesthetic effects were expressed as percent of control

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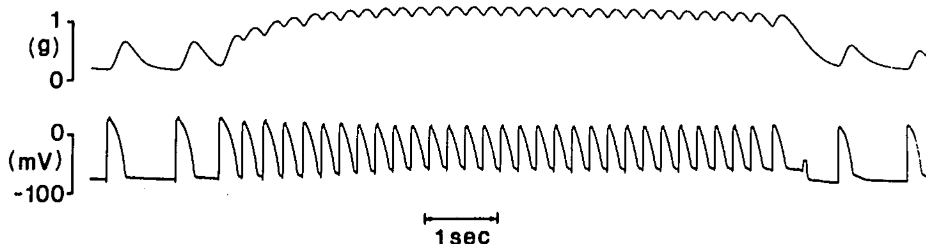

FIG. 1. Role of membrane potential in the development of myocardial tetanic contraction. Membrane potential (lower record) and developed force (upper record) during 4 Hz stimulation of rabbit papillary muscle treated with 1 μM ryanodine.

(no anesthetic) tetanic contraction strength and analyzed with an ANOVA followed by Dunnett's test (for comparisons within each anesthetic series) or Scheffe's F test (for comparisons between anesthetics) as appropriate. Significance was defined as $P < 0.05$. Anesthetic concentrations were chosen as those which produced approximately equal MAC multiples of isoflurane and halothane for the rabbit.[16]

Results

Characterization of Tetanus in Rabbit Papillary Myocardium

Rabbit papillary muscles are quiescent unless stimulated. In the presence of 1 μM ryanodine, an inhibitor of sarcoplasmic reticular function, the muscles respond to 4 Hz stimulation with development of nearly fused steady force (tetanus) within approximately 1.5 s (fig. 1). Developed force remains fairly steady until rapid stimulation is ceased. Concomitant records of membrane potential reveal that rapid stimulation produces an action potential that depolarizes only to approximately +10 mV and repolarizes to approximately −60 mV, a somewhat narrower voltage range than the +25 to −80 mV seen at slower stimulus rates. While slow (L-type) Ca channels, fast (T-type) Ca channels, and Na-Ca exchange must all be considered possible calcium carriers within this narrowed voltage range, Marban *et al.*[14] have provided convincing evidence using dihydropyridines to show that most of the Ca^{+2} entry during the tetanus in ferret occurs *via* the slow Ca channels. Similarly, in our preparation, 1 μM nifedipine significantly reduced tetanic contraction strength from 4.2 ± 0.7 g/mm^2 (control) to 0.6 ± 0.1 g/mm^2 (nifedipine treated) ($[Ca^{+2}]_{ext}$ = 2.5 mM; N = 4). The dependence of tetanic contraction upon extracellular calcium concentration is shown in figure 2. A proportionate increase in strength was observed at external calcium concentrations up to 2.5 mM with a plateau (saturation) above 5 mM. Marban *et al.*[14] have demonstrated using aequorin luminescence that internal calcium concentration continues to rise proportionate to external calcium concentration above the point at which the tetanic force has plateaued.

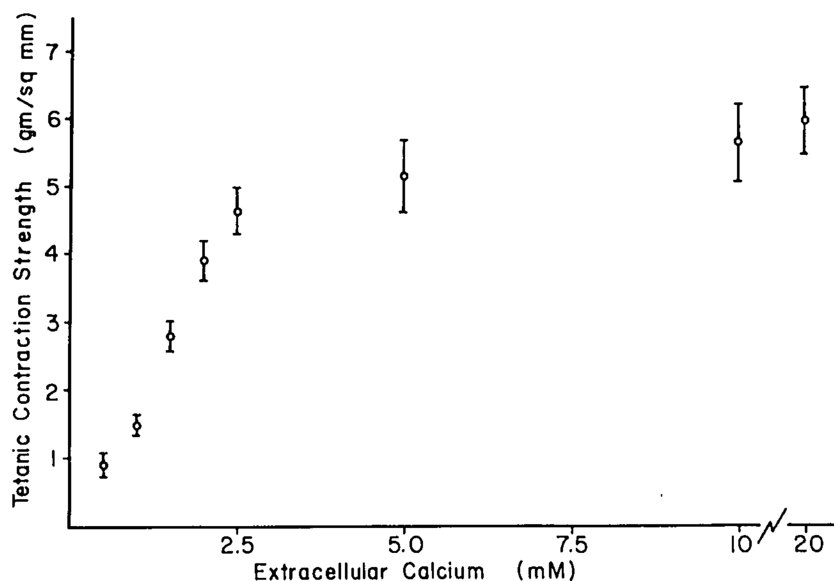

FIG. 2. Saturation of tetanic contraction strength with respect to extracellular calcium concentration. Error bars represent SEM (N = 4).

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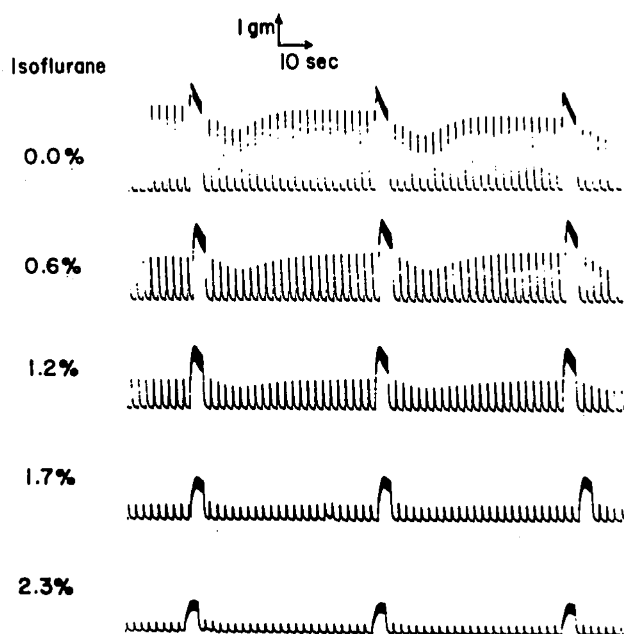

FIG. 3. Example of traces showing dose-dependent depression of tetanic contraction strength by 0.6, 1.2, 1.7, and 2.3% isoflurane. ([Ryanodine] = 1 μM, $[Ca^{+2}]_{ext}$ = 2.5 mM, muscle dia. = 0.93 mm).

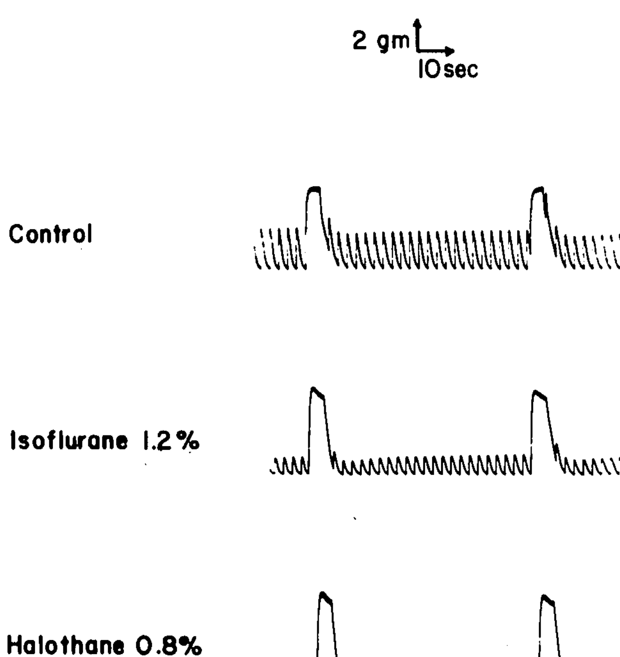

FIG. 5. Example of traces with $[Ca^{+2}]_{ext}$ = 20 mM demonstrating lack of effect of isoflurane 1.2% and halothane 0.8% on tetanic contractions. ([Ryanodine] = 1 μM, muscle dia. = 0.91 mm).

ANESTHETIC EFFECTS ON TETANIC CONTRACTION IN RABBIT PAPILLARY MYOCARDIUM

The effect of anesthetics on tetanic contraction strength at normal (2.5 mM) external calcium concentration was assessed in eight muscles using 0–2.3% isoflurane and in five muscles using 0–1.5% halothane. Typical traces from one such experiment with isoflurane are shown in figure 3. Tetanic force is represented by the maximum diastolic tension during the short periods of 4 Hz stimulus. Averaged results for each of the anesthetics are shown graphically in figure 4. Tetanic contractions were depressed significantly below control at all doses tested with both anesthetics in dose-dependent fashion. Differences between the effect of isoflurane and halothane at MAC-equivalent doses were not statistically significant.

In contrast to their effects at 2.5 mM external calcium concentration, neither 1.2% isoflurane nor 0.8% halothane depressed the strength of tetanic contractions at

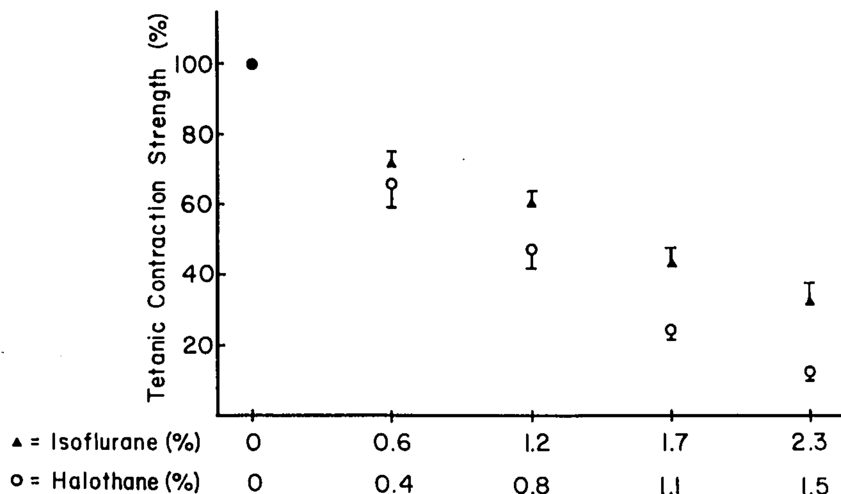

FIG. 4. Graphic representation of dose-dependent depression of tetanic contraction strength by isoflurane (0–2.3%) and MAC-equivalent doses of halothane (0–1.5%). At each concentration tested, tetanic contractions were significantly depressed from control. ([Ryanodine] = 1 μM, $[Ca^{+2}]_{ext}$ = 2.5 mM, N = 8 for isoflurane, N = 5 for halothane, error bars represent SEM).

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20 mM external calcium. This is shown by typical force traces in figure 5 and corresponding data in table 1.

Discussion

Results from these experiments demonstrate that sustained force tetani can be established in a rabbit papillary muscle preparation under very similar conditions to those used by Yue *et al.*[13] and Marban *et al.*[14] in the ferret. Membrane potential, response to variation in external calcium concentration, and response to the dihydropyridine calcium channel antagonist nifedipine all suggest that, as in the ferret, tetanic contractions are supported largely by the influx of calcium from outside the cell *via* the slow calcium channels. Marban *et al.*[14] has further demonstrated, using aequorin luminescence, that intracellular calcium concentration rises proportionate to external calcium concentration well above the level where plateau of force occurs. Thus, the plateau of tetanic force likely represents a myofibrillar calcium binding saturation as opposed to a calcium entry or cellular transit saturation. We have not reproduced the aequorin luminescence studies in our preparation because aequorin signals in the presence of anesthetics are of uncertain reliability.[17,18]

The sarcoplasmic reticulum of myocardial cells is involved both in the early triggered-release of calcium to the myofibrils and in rapid reuptake of calcium between beats.[2] Ryanodine inhibits normal sarcoplasmic reticular function,[15] likely by interaction with a specific sarcoplasmic reticular calcium channel protein.[19] Thus, in the presence of ryanodine, force development during rapid stimulation depends upon entry of extracellular calcium (with negligible intracellular release) and force is sustained in diastole as the sarcoplasmic reticulum fails to sequester calcium and sarcolemmal extrusion systems (*e.g.*, Na-Ca exchange, Ca-ATPase pump) are overwhelmed. Reiter *et al.*[20] have suggested that all calcium entering the myocardial cell must transit the sarcoplasmic reticulum, including that entering within the same beat. Since tetani can be established easily in the presence of ryanodine, it is clear that the ryanodine-sensitive sarcoplasmic reticulum is not involved in the transit of calcium entering from outside the cell. A hypothetical second component of sarcoplasmic reticulum that does not utilize ryanodine-sensitive calcium channels must be invoked to maintain Reiter's hypothesis[20]; however, such a component has yet to be demonstrated.

Our experiments with isoflurane and halothane suggest that both inhibit myocardial calcium influx measured by myofibrillar force development in tetanic contractions. If, as suggested by studies such as those of Rusy[21] and Su and Kerrick,[22] the myofibrillar response to calcium is unaffected by anesthetics, then it follows that both anesthetics likely inhibit the *net* extracellular calcium influx. (Interestingly, preliminary work by Housmans *et al.*[23] has reopened the question of a myofibrillar effect of the anesthetics, suggesting that halothane, enflurane, and isoflurane may perturb the load-variable calcium affinity of myofibrils.) Inhibition of tetanic contraction, however, *does not* prove direct competition at the slow calcium channel. Inhibition at any step involved in calcium influx (*e.g.*, slow channel entry, cellular transit, or, perhaps, myofibrillar binding) or acceleration of cellular calcium efflux could produce the observed results; the exact sites of interaction of the two anesthetics need not be identical. Resolution of these details must await more accurate measurements of cellular calcium transients. Whatever the sites, high calcium concentrations reverse the inhibition measured by tetanic contractions.

In conclusion, we have demonstrated that both isoflurane and halothane inhibit tetanic contractions, which are largely dependent upon the influx of extracellular calcium. Although exact mechanisms of inhibition of tetany are not identified, modulation of calcium influx *via* pathways independent of the ryanodine-sensitive sarcoplasmic reticulum likely plays a role. In a previous study,[6] we have shown that isoflurane also has no effect on the net calcium released from sarcoplasmic reticulum during potentiated-state contractions. Thus, it seems that the mechanism by which isoflurane produces negative inotropy is independent of measurable effects on classically described sarcoplasmic reticulum.

TABLE 1. Tetanic Contraction Strength (Percent of Control)

	$[Ca^{+2}]_{ext}$ = 2.5 mM	$[Ca^{+2}]_{ext}$ = 20 mM
Isoflurane 1.2%	60 ± 4*	101 ± 11
Halothane 0.8%	47 ± 6*	91 ± 17

* Results significantly different than control.

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