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Halothane Increases Smooth Muscle Protein Phosphatase in Airway Smooth Muscle

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Background: **Halothane relaxes airway smooth muscle, in part, by decreasing the force produced for a given intracellular [Ca^{2+}] (*i.e.*, Ca^{2+} sensitivity) during muscarinic stimulation, an effect produced by a decrease in regulatory myosin light-chain (rMLC) phosphorylation. The authors tested the hypothesis that halothane reduces rMLC phosphorylation during muscarinic stimulation at constant intracellular [Ca^{2+}] by increasing smooth muscle protein phosphatase (SMPP) activity, without changing myosin light-chain kinase (MLCK) activity.**

Methods: **Enzyme activities were assayed in β-escin permeabilized strips of canine tracheal smooth muscle. Under conditions of constant intracellular [Ca^{2+}], the rate of rMLC phosphorylation was measured by Western blotting during inhibition of SMPP with microcystin-LR (to assay MLCK activity) or during inhibition of MLCK by wortmannin and adenosine triphosphate depletion (to assay SMPP activity). The effect of halothane (0.8 mM) on enzyme activities and isometric force during stimulation with 0.6 μM Ca^{2+} and 10 μM acetylcholine was determined.**

Results: **Halothane produced a 14 ± 8% (mean ± SD) decrease in isometric force by significantly reducing rMLC phosphorylation (from 32 ± 9% to 28 ± 9%). Halothane had no significant effect on any parameter of a monoexponential relation fit to the data for the MLCK activity assay. In contrast, halothane significantly decreased the half-time for rMLC dephosphorylation in the SMPP activity assay (from 0.74 ± 0.28 min to 0.44 ± 0.10 min), indicating that it increased SMPP activity.**

Conclusions: **Halothane decreases Ca^{2+} sensitivity and rMLC phosphorylation in airway smooth muscle during muscarinic receptor stimulation by increasing SMPP activity, without affecting MLCK, probably by disrupting receptor G-protein signaling pathways that inhibit SMPP.**

HALOTHANE and other volatile anesthetics directly relax airway smooth muscle, in part, by decreasing the amount of force produced for a given intracellular [Ca^{2+}] (*i.e.*, Ca^{2+} sensitivity) during muscarinic stimulation.[1–4] Force production in smooth muscle is controlled by the phosphorylation of the regulatory myosin light-chain (rMLC), which increases actomyosin adenosine triphosphate (ATP)ase activity and force.[5] rMLC phosphorylation depends on the balance between the activities of myosin light-chain kinase (MLCK) and smooth muscle protein phosphatase (SMPP) (fig. 1). MLCK activity is regulated by the binding of calcium–calmodulin complexes in response to increased intracellular [Ca^{2+}] produced by receptor stimulation, favoring increased phosphorylation of rMLC.[6] rMLC phosphorylation can also increase if the activity of SMPP is inhibited. Such inhibition, mediated *via* a cascade of both heterotrimeric and small monomeric guanine binding proteins (G proteins), is primarily responsible for agonist-induced increases in Ca^{2+} sensitivity in smooth muscle.[7–9] Ca^{2+} sensitivity could also be increased by G-protein–mediated regulation of MLCK, which can itself be phosphorylated at a specific site that regulates its activity.[10] Finally, recent studies suggest that a kinase activated by G proteins (rho-associated kinase) can also directly phosphorylate rMLC,[11,12] providing another putative means by which receptor activation could increase Ca^{2+} sensitivity.

We have shown that halothane inhibits Ca^{2+} sensitivity of canine tracheal smooth muscle (CTSM) during muscarinic stimulation by decreasing rMLC phosphorylation.[1] This decrease could be caused by a reduction in MLCK activity, an increase in SMPP activity, or a combination of both factors. Halothane has no effect on Ca^{2+} sensitivity of rMLC phosphorylation in the absence of receptor stimulation,[4,14,15] indicating that it does not directly affect the activities of MLCK or SMPP. Rather, based on current understanding of the regulation of rMLC phosphorylation and our prior experimental results, we have suggested that halothane interferes with the activation of a heterotrimeric G protein linked to the muscarinic receptor, possibly by inhibiting its dissociation.[15] Halothane-induced inhibition of the G-protein pathway that normally reduces SMPP activity in response to muscarinic stimulation would decrease rMLC phosphorylation.

The purpose of this study was to test the hypothesis that halothane reduces rMLC phosphorylation under conditions of constant intracellular [Ca^{2+}] in airway smooth muscle during muscarinic stimulation by increasing SMPP activity, without changing MLCK activity. To evaluate this hypothesis, we developed and validated *in situ* assays of MLCK and SMPP activity in CTSM permeabilized with β-escin.

Materials and Methods

Tissue Preparation

After obtaining approval from the Institutional Animal Care and Use Committee at the Mayo Clinic and Mayo Foundation, mongrel dogs (15–20 kg) of either sex were anesthetized with an intravenous injection of pentobar-

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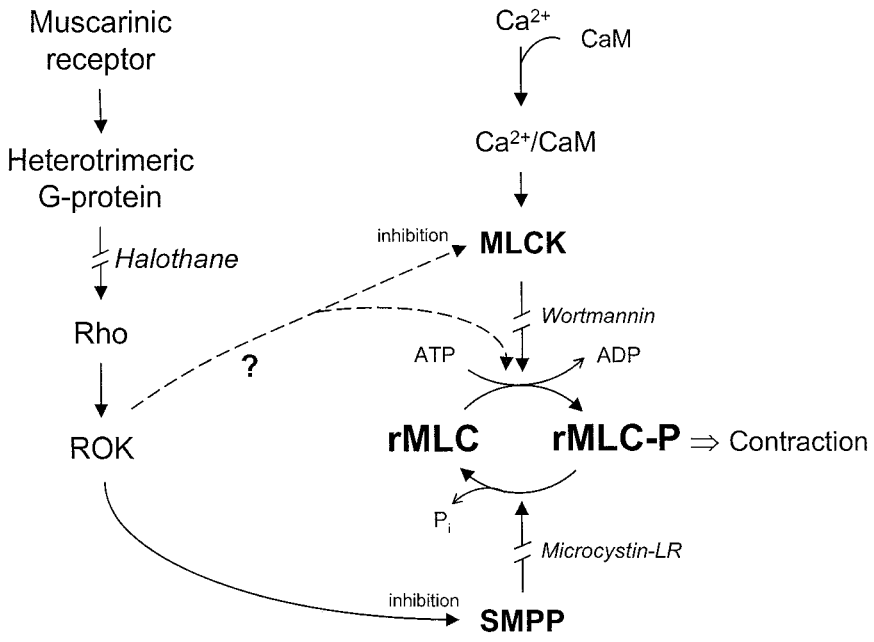

Fig. 1. A model of signal transduction of acetylcholine-induced Ca^{2+} sensitization in smooth muscle. Rho = monomeric G protein; ROK = rho-associated kinase; CaM = calmodulin; MLCK = myosin light-chain kinase; rMLC = regulatory myosin light chain; SMPP = smooth muscle protein phosphatase. Dashed lines indicate pathways that are of uncertain physiologic significance. Also shown is the putative site of halothane action and the actions of compounds used to perform MLCK and SMPP assays (wortmannin and microcystin-LR).

bital sodium (30 mg/kg) and exsanguinated. The trachea was excised and immersed in chilled physiologic salt solution of the following composition: 110.5 mM NaCl, 25.7 mM $NaHCO_3$, 5.6 mM dextrose, 3.4 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 0.8 mM $MgSO_4$. Fat, connective tissue, and the epithelium were removed with tissue forceps and scissors under microscopic observation to make muscle strips of 0.1-00.2-mm width, 1-cm length, and 0.2–0.3-mg wet weight. To measure isometric force, the strips were mounted in 0.1-ml cuvettes and continuously superfused with physiologic saline solution (PSS) (37°C) aerated with 94% O_2–6% CO_2. One end of the strips was anchored *via* stainless steel microforceps to a stationary metal rod and the other end *via* stainless steel microforceps to a calibrated force transducer (model KG4; Scientific Instruments, Heidelberg, Germany). Each strip was stretched to optimal length for isometric force as previously described.[4]

Permeabilization Procedure

Muscle strips were permeabilized with β-escin[16] by a method validated for canine tracheal smooth muscle in our laboratory.[4] 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. Muscle strips were superfused for 20 min with relaxing solution (*i.e.*, low [Ca^{2+}]) containing 100 μM β-escin. The relaxing solution was made up in the following composition using the algorithm of Fabiato and Fabiato[17]: 7.5 mM MgATP, 4 mM 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 the temperature studied with KOH or HCl. After the permeabilization procedure, strips were washed with relaxing solution without β-escin for 10 min. 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 intracellular [Ca^{2+}] constant. Solutions of varying free Ca^{2+} concentrations used in the subsequent experiment were also prepared using the Fabiato algorithm.

Regulatory Myosin Light Chain Phosphorylation Measurements

Strips for rMLC phosphorylation measurements were separately prepared according to the same procedures as force measurements but incubated in wells at approximately optimal length instead of being superfused.[18] Preliminary experiments revealed that muscle length does not affect rMLC phosphorylation under these conditions (data not shown). After an equilibration period of 30 min in aerated PSS at 25°C, the strips were incubated in Ca^{2+}-free PSS containing 2 mM EGTA for 15 min. Intracellular Ca^{2+} stores were then depleted by exposing the strips to 10 μM acetylcholine for 10 min. Acetylcholine was removed from the bath by exchanging solutions repeatedly with Ca^{2+}-free PSS over 5 min before tissues were permeabilized. After experimental interventions, muscle strips were flash-frozen by rapid immersion in acetone containing 10% trichloroacetic acid and 10 mM dithiothreitol cooled to −80°C with crushed dry ice. Strips were then allowed to warm to room temperature in the same solution. After washing out trichloroacetic acid with acetone–dithiothreitol, strips were allowed to dry. rMLC was extracted and phosphorylation was determined by glycerol–urea gel electrophoresis followed by Western blotting. Unphosphorylated and

phosphorylated bands of rMLC were visualized by phosphorimage analysis (Cyclone; Packard Instrument Co., Meriden, CT), and fractional phosphorylation was calculated as the density ratio of the sum of monophosphorylated and diphosphorylated rMLC to total rMLC using OptiQuaNT software (Packard Instrument Co.).

Administration of Halothane

Halothane was delivered to solutions *via* a calibrated vaporizer. Concentrations of halothane in solutions bathing the strips were determined by gas chromatography from samples obtained at the end of the protocol using an electron capture detector (model 5880A; Hewlett-Packard, Waltham, MA) according to the method of Van Dyke and Wood.[19]

Experimental Protocols

Effect of Halothane on Calcium Sensitivity. To confirm that halothane significantly affected acetylcholine-induced calcium sensitization, one pair of permeabilized strips was prepared from each dog. All strips were first contracted with 10 μM Ca^{2+}; subsequent force measurements were normalized to these maximal contractions. After washout, the strips were then stimulated with 0.6 μM Ca^{2+}. After 10 min, 10 μM acetylcholine was added to the solutions. All solutions with acetylcholine in this and subsequent protocols also included 10 μM guanosine triphosphate (GTP) to support G-protein function. After another 10 min, halothane was added to the superfusate of one strip for 15 min. The other strip served as a control for the effects of time. These experiments were performed at 25°C.

Smooth Muscle Protein Phosphatase Assay. We adapted an *in situ* assay of SMPP activity described in other types of smooth muscle[9,20-22] to the airway. The principle of this assay is to rapidly inhibit MLCK activity by removing ATP from solutions, bathing the strip, and adding wortmannin, an MLCK inhibitor. The rate of the subsequent dephosphorylation of rMLC by SMPP is an index of its activity.

The assay was first validated by determining the effects of acetylcholine stimulation on SMPP activity. According to the current model of smooth muscle contractile regulation,[8] muscarinic stimulation should inhibit SMPP activity *via* a process mediated by G-proteins. Thirteen strips were prepared from each dog and maintained at 25°C. After permeabilization, one strip was frozen while in relaxing solution (baseline). All other strips were stimulated with 3.2 μM Ca^{2+} for 15 min; half of the strips also received 10 μM acetylcholine. To begin the assay, the strips were then exposed to a rigor solution (relaxing solution without MgATP, creatine phosphatase, creatine phosphokinase, or free Mg^{2+}, substances that are necessary for crossbridge cycling) with 10 μM wortmannin. Strips were frozen for rMLC measurements immediately before and at 0.5, 1, 3, 5, and 10 min after exposure to rigor solution.

To determine the effects of halothane on SMPP activity, a set of 13 permeabilized strips was first stimulated with 0.6 μM Ca^{2+} for 10 min, and then 10 μM acetylcholine was added for an additional 10 min. In half of the strips, the solution was then aerated with halothane in air for the remainder of the experiment. After an additional 15 min, all strips were exposed to rigor solution with wortmannin. Strips were frozen for rMLC measurements as described above.

We used two different [Ca^{2+}] in the two experiments to optimize conditions according to the experimental goals. Because of the variability inherent in rMLC phosphorylation measurements in permeabilized strips, it is desirable that the initial rMLC phosphorylation at the time of assay are as high as possible. A relatively high [Ca^{2+}] (3.2 μM) was used in the first protocol to maximize initial rMLC phosphorylation, a factor especially important in the absence of acetylcholine. However, our prior work[4,14] and preliminary experiments show that at high [Ca^{2+}], halothane has a relatively small effect on Ca^{2+} sensitivity. Thus, studies determining the effect of halothane were performed under lower [Ca^{2+}] (0.6 μM) in which the presence of acetylcholine provided sufficient initial rMLC phosphorylation values to perform the assay.

Myosin Light-Chain Kinase Assay. The principle of this assay is to first inhibit SMPP (by exposure to microcystin-LR) in a rigor solution, then rapidly activate MLCK by adding Ca^{2+}, MgATP, creatine phosphatase, and creatine phosphokinase.[9,23] The rate of the subsequent phosphorylation of rMLC by MLCK is an index of its activity.

Ten strips were prepared from each dog and maintained at 25°C. After permeabilization, the solutions bathing half of the strips were aerated with halothane in air for the remainder of the experiment. The strips were washed several times with rigor solution to remove ATP from the preparation. Microcystin-LR (10 μM) and 10 μM acetylcholine was then added to the rigor solution, compounds that remained a component of all subsequent solutions. We confirmed in preliminary studies that this concentration of microcystin-LR prevented dephosphorylation of rMLC under these conditions (see Discussion). Beginning 4 min later, the strips were exposed to 0.6 μM Ca^{2+}. One minute after this, MLCK was activated by adding 7.5 mM MgATP, 10 mM phosphocreatine, and 0.1 mg/ml creatine kinase. Strips were frozen for rMLC measurements immediately before and at 0.5, 1, 1.5, 2, and 5 minutes after exposure to ATP and enzymes.

To validate this assay, the experiments were repeated with tissue from another series of dogs with the strips maintained at a lower temperature (15°C), which should decrease MLCK activity.

Materials

The polyclonal affinity-purified rabbit anti-20-kDa rMLC antibody was a generous gift of Dr. Susan J. Gunst

(Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, IN). ATP disodium salt was purchased from Research Organics (Cleveland, OH). All other drugs and chemicals were purchased from Sigma Chemical (St. Louis, MO). A23187 was dissolved in dimethyl sulfoxide (0.05% final concentration). All other drugs and chemicals were prepared in distilled filtered water.

Statistical Analysis

For both assays, data from individual experiments were fit to exponential equations using nonlinear regression (Sigma Stat; Jandel, San Rafael, CA). For the SMPP assays, the relation between rMLC phosphorylation (expressed as a percentage of the initial value) and time was fit to the following equation:

$$P = P_o + ae^{-bt} \quad (1)$$

where P is rMLC phosphorylation, P_o is the rMLC phosphorylation at the beginning of the assay, t is time, and a and b are coefficients representing the amount and rate of rMLC phosphorylation change, respectively, during the assay. For the MLCK assay, the relation between rMLC phosphorylation and time was fit to the following equation:

$$P = P_o + a(1 - e^{-bt}) \quad (2)$$

with coefficients defined as in equation 1.

Equation coefficients were then compared using paired or unpaired *t* tests as appropriate. This procedure was successful (regression coefficient of determination > 0.85 for each individual experiment) with the exception of the SMPP activity data obtained during acetylcholine stimulation with 3.2 μM Ca^{2+}, in which meaningful coefficients could not be calculated for two experiments. Thus, for this experiment (which validated the effect of acetylcholine on SMPP activity), a different analysis was performed. Nonlinear regression was applied to the pooled data (*i.e.*, all data for each condition from individual experiments taken together) according to the technique described by Meddings *et al.*[24] This technique is more robust for analysis of outlying data but sacrifices the benefits of paired analysis.

To confirm the qualitative results of these analyses, simple half-times for decreases in relative rMLC phosphorylation for each SMPP assay experiment were also calculated by linear interpolation of the relation between rMLC phosphorylation and time.

When isometric force was measured, it was expressed as percentage of the maximal force induced by 10 μM Ca^{2+} determined in each individual strip before the experimental protocol. Relaxation was expressed as a percent of the initial force (before exposure to halothane), adjusted for the effect of time using the change in force of the time-matched control strip as previously described.[15] Statistical assessments were made by paired *t* test. *P* less than 0.05 was considered significant. Data are expressed as mean ± SD; n represents the number of dogs.

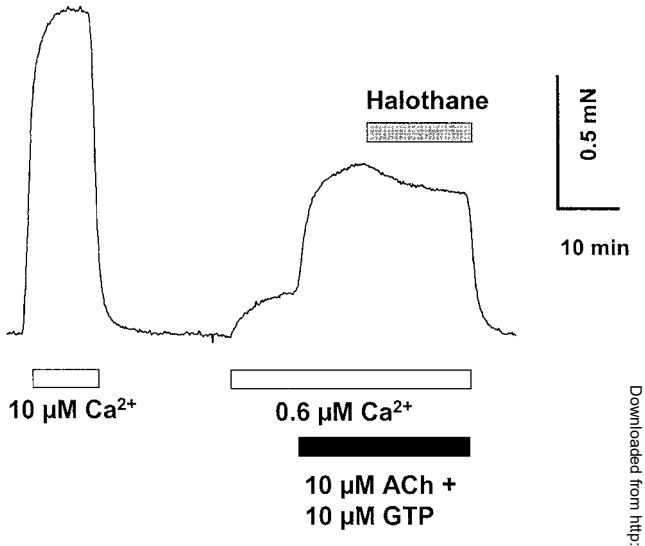

Fig. 2. Representative tracing showing the effect of halothane (0.8 mM) on isometric force in a β-escin permeabilized canine tracheal smooth muscle strip stimulated with calcium, acetylcholine (ACh), and guanosine triphosphate (GTP).

Results

Effect of Halothane on Calcium Sensitivity

Exposure of permeabilized strips to 0.6 μM Ca^{2+} increased force to 27 ± 4% of the initial response to 10 μM Ca^{2+} (maximal force; n = 6; fig. 2). Addition of 10 μM acetylcholine further increased force to 65 ± 8% of maximal force, indicating that acetylcholine increased calcium sensitivity. Exposure of the acetylcholine-stimulated strips to halothane (0.6 ± 0.1 mM) produced a 14 ± 8% relaxation, demonstrating that halothane attenuates acetylcholine-induced increases in Ca^{2+} sensitivity under the conditions of the SMPP and MLCK activity assays.

Smooth Muscle Protein Phosphatase Activity

The first experiment examined the effect of acetylcholine on SMPP activity. Acetylcholine (10 μM) significantly increased the rMLC phosphorylation measured during exposure to 3.2 μM Ca^{2+} (from 39 ± 9% to 48 ± 11%; $P < 0.001$; n = 7; fig. 3A). Exposure to assay conditions (wortmannin and rigor solution at time 0; fig. 3) caused a decrease in rMLC phosphorylation, which reached values not significantly different from baseline (measured before exposure to increased [Ca^{2+}]) within 10 min (fig. 3A). Acetylcholine significantly increased the half-time for decreases in relative rMLC phosphorylation (from 0.6 ± 0.4 min to 2.8 ± 2.2 min; $P < 0.04$; fig. 3B), indicating that acetylcholine decreased SMPP activity. Analysis by nonlinear regression of the pooled data gave similar results. Acetylcholine significantly decreased the relative rate of decline in rMLC phosphory-

lation, as shown by a decrease in the equation coefficient b from 1.45 ± 0.69 min^{-1} to 0.52 ± 0.39 min^{-1} ($P < 0.04$). Other parameters of the regression equation were not affected (data not shown).

The second experiment examined the effects of halothane on SMPP activity during muscarinic stimulation. Halothane (0.8 ± 0.2 mM, equivalent to approximately 3 minimum alveolar concentration) significantly decreased the rMLC phosphorylation measured during exposure to 0.6 μM Ca^{2+} and 10 μM acetylcholine (from 32 ± 9% to 28 ± 9%; $P < 0.001$; n = 7; fig. 4A). Exposure to assay conditions produced a decrease in rMLC phosphorylation, which reached values not significantly different from baseline within 2 min. Halothane significantly decreased the half-time for the decline in relative rMLC phosphorylation (from 0.74 ± 0.28 min to 0.44 ± 0.11 min; $P < 0.02$; fig. 4B), indicating that halothane increased SMPP activity. This pattern was also present when the data were analyzed using nonlinear regression of individual experiments. Halothane significantly increased the relative rate of decline in rMLC phosphorylation, as shown by an increase in the coefficient b (from equation 1) from 1.25 ± 0.49 min^{-1} to 2.36 ± 0.79 min^{-1} ($P < 0.005$). Other parameters of the regression equation were not affected (data not shown).

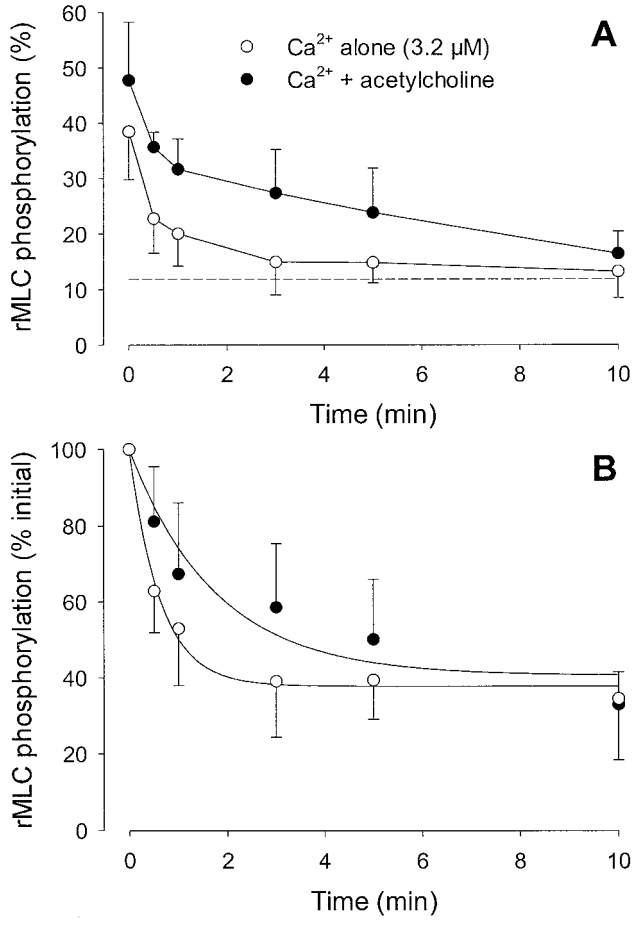

Fig. 3. Relation between regulatory myosin light-chain (rMLC) phosphorylation and time during smooth muscle protein phosphatase assay conditions (adenosine triphosphate depletion with wortmannin) during exposure to either Ca^{2+} alone (3.2 μM) or Ca^{2+} (3.2 μM) with 10 μM acetylcholine and 10 μM guanosine triphosphate (n = 7). Values are mean ± SD and are expressed as absolute values (*A*) or as a percentage of initial values (*B*). Dashed line in (*A*) denotes the mean baseline phosphorylation value (before exposure to calcium) of 11 ± 4%. Lines in the lower panel represent nonlinear regression of mean values fit to a monoexponential relation (equation 1).

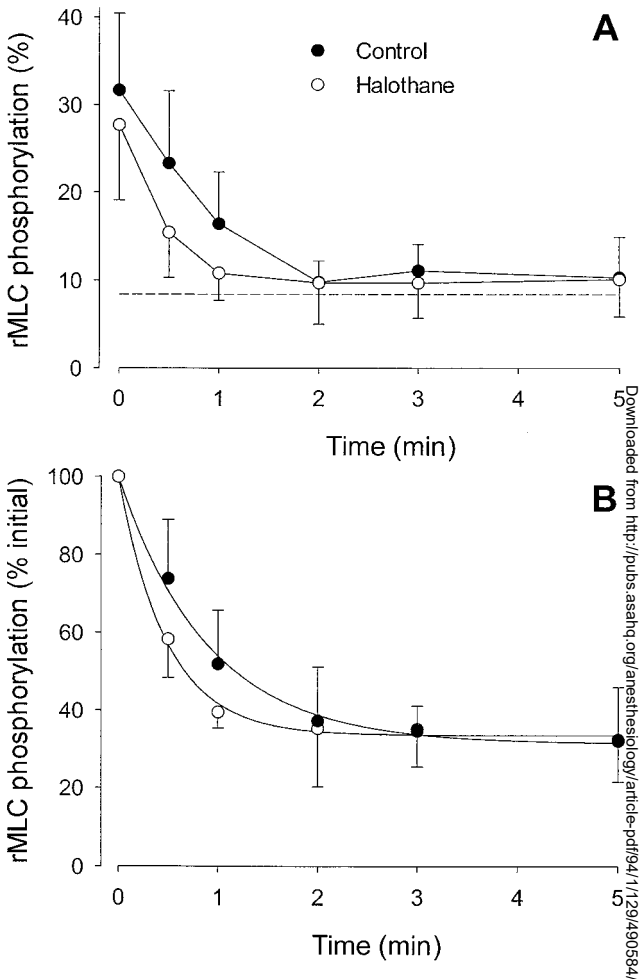

Fig. 4. Relation between regulatory myosin light-chain (rMLC) phosphorylation and time during smooth muscle protein phosphatase assay conditions (adenosine triphosphate depletion with wortmannin) during exposure to 0.6 μM Ca^{2+}, 10 μM acetylcholine, and 10 μM guanosine triphosphate with (Halothane) or without (Control) halothane (n = 7). Values are mean ± SD and are expressed as absolute values (*A*) or as a percentage of initial values (*B*). Dashed line in (*A*) denotes the mean baseline phosphorylation value (before exposure to calcium) of 8 ± 2%. Lines in the lower panel represent nonlinear regressions of mean values fit to a monoexponential relation (equation 1).

Myosin Light-Chain Kinase Assay

Assay conditions (activation of MLCK by the addition of MgATP) produced a sustained increase in rMLC phosphorylation (fig. 5). The time course of this increase was well described by a single exponential relation. Decreasing the temperature at which the assay was performed significantly decreased the time coefficient b (from equation 2; $P <$

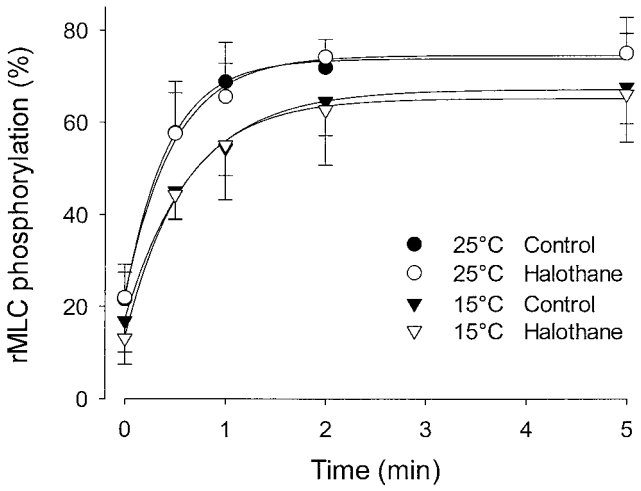

Fig. 5. Relation between regulatory myosin light-chain (rMLC) phosphorylation and time during myosin light-chain kinase assay conditions (exposure to microcystin-LR) during exposure to 0.6 μM Ca^{2+}, 10 μM acetylcholine, and 10 μM guanosine triphosphate with (Halothane) or without (Control) halothane at 25°C (n = 5) and 15°C (n = 6). Values are mean ± SD. Lines represent nonlinear regression of mean values fit to a monoexponential relation (equation 2).

0.03; table 1), showing that the decrease in temperature decreased MLCK activity. Halothane (0.8 ± 0.1 mM at 15°C and 0.8 ± 0.2 mM at 25°C) did not affect any coefficient of the fitted equation at either temperature (table 1), indicating that halothane did not affect MLCK activity.

Discussion

The major finding of this study is that halothane decreases Ca^{2+} sensitivity and rMLC phosphorylation in airway smooth muscle during muscarinic receptor stimulation by increasing SMPP activity, without affecting MLCK activity.

Table 1. Regression Coefficients for MLCK Assay

Temperature (°C)	Coefficient	Control	Halothane
25 (n = 5)	P_o (%)	21 ± 3	22 ± 3
	a (%)	52 ± 3	53 ± 3
	b (min^{-1})	2.8 ± 0.5	2.8 ± 0.8
15 (n = 6)	P_o (%)	17 ± 3	12 ± 2
	a (%)	50 ± 2	54 ± 5
	b (min^{-1})	1.6 ± 0.2*	1.9 ± 0.1

Values are mean ± SD.

Coefficients are obtained from nonlinear regressions fit to the equation

$$P = Po + a(1 - e^{-bt}),$$

where P is regulatory myosin light chain (rMLC) phosphorylation, P_o is the rMLC phosphorylation at the beginning of the assay, t is time, and a and b are coefficients representing the amount and rate of rMLC phosphorylation increase, respectively, during the assay.

* Significant difference from corresponding value at 25°C, unpaired *t* test, $P < 0.05$.

MLCK = myosin light chain kinase.

Myosin Light-Chain Kinase Activity

Actomyosin ATPase activity (and hence, cross-bridge cycling rate and force production) in smooth muscle is regulated primarily by the phosphorylation of the serine-19 and threonine-18 residues of the rMLC.[25] MLCK, activated by the binding of Ca^{2+}–calmodulin, catalyzes phosphorylation of these residues. MLCK activity can be assayed using the purified enzyme or *in situ* by measuring the rate of substrate phosphorylation (*i.e.*, rMLC) in the absence of opposing SMPP activity. The latter method has the advantage of preserving intracellular mechanisms that may regulate MLCK activity (see below).[9,23] Its validity depends on abolition of SMPP activity under the conditions of the assay, achieved in our experiments with microcystin-LR. We confirmed in preliminary experiments that inhibition was complete by first performing the assay (producing maximal rMLC phosphorylation), then inhibiting MLCK activity by removing calcium and ATP from the solutions (while maintaining exposure to microcystin-LR). If significant SMPP activity was still present, then rMLC phosphorylation would decrease. However, rMLC phosphorylation did not change under these conditions (data not shown), indicating that microcystin-LR completely inhibited SMPP activity. We further confirmed the validity of the assay by measuring the effect of temperature on MLCK activity. The ratio of activities at 25°C and 15°C (the Q_{10}), calculated using the coefficient b in equation 1, was 1.8, very similar to the value for MLCK reported in other types of smooth muscle.[26,27]

There are two potential mechanisms by which muscarinic activation of G proteins might affect kinase activity directed toward the rMLC independently of changes in intracellular $[Ca^{2+}]$ (fig. 1). First, MLCK activity *in situ* is regulated through phosphorylation of a regulatory site (site A) by Ca^{2+}–calmodulin-dependent protein kinase II in response to increases in intracellular $[Ca^{2+}]$.[10] Phosphorylation of MLCK desensitizes the enzyme to activation by Ca^{2+} and thus serves as a mechanism for limiting MLCK activation in response to increases in $[Ca^{2+}]$.[28] There is limited evidence that receptor-linked mechanisms may also phosphorylate this site and regulate MLCK activity. Tang *et al.*[29] found that GTPγS, which directly stimulates G proteins, reduced MLCK activity in tracheal smooth muscle. They suggested that activated G proteins inhibit phosphatases that dephosphorylate MLCK, an action that would decrease MLCK activity. However, other studies of airway[30] and vascular[9] smooth muscle did not find an effect of GTPγS on assays of rMLC phosphorylation. Second, recent studies indicated that the rMLC may also be directly phosphorylated by rho-associated kinase (ROK),[12] which is activated during muscarinic stimulation (see discussion below). If this factor is significant in CTSM, the rate of rMLC phosphorylation under assay conditions would reflect the activity of both MLCK and ROK. However, the lack of effect of

G-protein activation by GTPγS on the rMLC phosphorylation assays[9,30] does not support this action. Furthermore, Iizuka *et al.*[31] found no evidence that ROK directly phosphorylates rMLC in rabbit tracheal smooth muscle, and other experimental evidence is also not supportive of this possibility.[32,33] Thus, there is little evidence that any direct phosphorylation of rMLC by ROK contributes to contraction in smooth muscle.

If halothane reduced rMLC phosphorylation by inhibiting MLCK activity, it could either directly inhibit its activity (by an action on the enzyme itself) or modulate any receptor-linked regulatory mechanisms (if present). The present findings provide no evidence to support either of these actions. The lack of halothane effect on MLCK activity is consistent with our prior observation that halothane has no effect on rMLC phosphorylation or Ca^{2+} sensitivity in the absence of receptor stimulation and confirms that halothane does not directly inhibit MLCK.[2,4,14,15] Furthermore, if receptor-linked mechanisms regulate MLCK activity in CTSM by means other than changing intracellular [Ca^{2+}] (a question not specifically addressed in this study), then these mechanisms are not affected by halothane.

Smooth Muscle Protein Phosphatase Activity

Several serine–threonine protein phosphatases are present in smooth muscle. rMLC phosphorylation is regulated primarily by the type 1 phosphatase SMPP-1M, a heterotrimer consisting of a regulatory subunit that binds to myosin, a catalytic subunit, and a smaller subunit of unknown function.[34,35] Phosphorylation of a site on the myosin binding subunit (threonine 695) inhibits SMPP activity.[36,37] This site is phosphorylated by ROK, which is activated by the GTP-bound form of the monomeric G protein rhoA.[36] According to current understanding (fig. 1),[38] rhoA itself is activated by a heterotrimeric G protein coupled to muscarinic receptors. Thus, muscarinic receptor stimulation activates a cascade of G proteins that ultimately inhibits SMPP activity, thus increasing rMLC phosphorylation and Ca^{2+} sensitivity. We have provided support for this model in CTSM by demonstrating that inhibition of rhoA by adenosine diphosphate–ribosylation produced by exotoxin C3 inhibits acetylcholine-induced calcium sensitization[15]; a subsequent study also supports its validity in rabbit tracheal smooth muscle.[31]

To assess the regulation of SMPP by this complex system, SMPP activity must be assayed *in situ* rather than by using purified enzyme.[9,20–22,33] Tissue homogenates have also been used,[23,30] although homogenization disrupt features such as receptor G-protein interactions. The interpretation of *in situ* assays depends on the assumption that MLCK activity is blocked under the conditions of the assay, exposure to rigor solutions (*i.e.*, no ATP) containing wortmannin, a kinase inhibitor. We have previously shown that wortmannin completely blocks force and actomyosin ATPase activity at these calcium concentrations even in the presence of ATP.[39] As predicted, activation of G proteins by muscarinic stimulation decreased SMPP activity and thus increased rMLC phosphorylation.

Before halothane effects are discussed, two features of the assay are worth noting. First, rMLC phosphorylation did not fall below baseline values measured with initial low [Ca^{2+}] conditions, even in the presence of unopposed SMPP activity, a finding noted in several prior studies using this technique.[20–23,33] In addition to serine-19 and threonine-18, there are several other sites on rMLC that can be phosphorylated (*e.g.*, serine-1, serine-2, and threonine-9).[25] We suggest that these sites are phosphorylated under baseline conditions and that their phosphorylation remains constant during stimulation and assay conditions. This explanation is consistent with the fact that these sites are not good substrates for SMPP-1M,[40] do not play a significant role in the regulation of actomyosin ATPase activity in response to muscarinic stimulation,[25] and are not phosphorylated by muscarinic stimulation in CTSM.[41] Thus, their presence should not affect the interpretation of assay kinetics. Second, the kinetics of the assay measured during acetylcholine stimulation appeared to depend on [Ca^{2+}], a factor that has not been previously studied. During exposure to 10 μM acetylcholine, SMPP activity was significantly lower at 3.2 μM Ca^{2+} compared with 0.6 μM Ca^{2+} (half-times for decreases in relative rMLC phosphorylation of 2.8 ± 2.2 min and 0.74 ± 0.28 min, respectively; $P < 0.03$ by unpaired *t* test; compare figs. 3 and 4). In addition, the data at high [Ca^{2+}] were sometimes not well described by a first-order process, as seen by the deviation of data from a monoexponential relation in figure 3B. Most other studies have also found that dephosphorylation of the rMLC measured during the SMPP assay is not well fit by apparent first-order kinetics.[20–23,33] Considering the complexity of the underlying regulatory mechanisms operating during muscarinic stimulation, many of which may be themselves modulated by Ca^{2+}, this Ca^{2+} dependence of SMPP kinetics, although of unknown origin, is perhaps not surprising and worthy of future study. Because comparisons within each experiment were made under conditions of constant [Ca^{2+}], this factor does not affect the interpretation of the effects of acetylcholine or halothane.

As hypothesized, halothane increased SMPP activity and thus decreased rMLC phosphorylation during muscarinic stimulation. Because halothane has no effect on rMLC phosphorylation or Ca^{2+} sensitivity in the absence of receptor stimulation,[2,4,14,15] it must interfere with receptor-mediated inhibition of SMPP rather than directly stimulate SMPP activity. According to our previous findings, halothane inhibits the function of the heterotrimeric G protein coupled to the muscarinic receptor, possibly by preventing its dissociation in response to

receptor activation (fig. 1).[15] Impairment of G-protein function would decrease phosphorylation of the regulatory subunit of SMPP, thus increasing its activity and reducing rMLC phosphorylation. As we have noted in previous work, anesthetics relax airway smooth muscle by multiple mechanisms, the relative importance of which depends on the level of contraction.[2] Based on current understanding, the actions of halothane on calcium sensitivity would be particularly important during maximal stimulation, such as that present during severe bronchoconstriction.

In summary, halothane decreases Ca^{2+} sensitivity and rMLC phosphorylation in airway smooth muscle during muscarinic receptor stimulation by increasing SMPP activity, without affecting MLCK activity. In addition to providing insights into anesthetic mechanism of action in smooth muscle, these results, when taken together with our prior work, demonstrate that anesthetics may affect enzymes *in situ* by affecting systems that regulate their activity, rather than by a direct effect on the enzyme itself.

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