

The Role of Airway Smooth Muscle during an Attack of Asthma Simulated *In Vitro*

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Excessive narrowing of airways in response to contractile agonists is a characteristic feature of asthma. We hypothesized that airway smooth muscle (ASM) adaptation to short lengths could contribute to exaggerated airway narrowing during an acute attack of asthma by allowing the muscle to regain its ability to generate maximal force at a shortened length. To test this hypothesis we mimicked, *in vitro*, the sequence of contractile events that would occur during a spontaneous attack of asthma. Trachealis muscle was challenged with carbachol (300 nM, submaximal dose) and allowed to shorten to approximately half of its original length. After 30 min of adaptation at the shortened length in the presence of carbachol, muscle force, amount and rate of shortening in response to electrical stimulation were compared with corresponding values obtained from control experiments during which the ASM was not adapted to the short length. After adaptation at the shortened length the developed force, amount and rate of shortening increased by 1.93 ± 0.08 -, 1.57 ± 0.12 -, and 1.75 ± 0.2 -fold, respectively. Shortening of ASM in response to contractile agonists can lead to adaptation of the muscle to the shortened length that, in turn, can result in further shortening and the potential for airway closure.

Keywords: length adaptation; muscle force; shortening velocity; trachealis

Asthmatic airways *in vivo* narrow excessively in response to a number of stimuli, including dry air, hypo- and hypertonic aerosols, cholinergic agonists, histamine, and allergens (1–3). Excessive airway narrowing could be related to an increased amount of airway smooth muscle (ASM) (4–8), which would allow the development of greater wall stress and an increased ability to narrow against opposing loads. Alternatively, greater ASM shortening could be due to an alteration in muscle phenotype so that the same amount of muscle can generate greater force and shortening than normal ASM (9). However, most investigations of asthmatic ASM *in vitro* have not reported greater ASM force, active stress, or shortening than control ASM (for a review, see Ref. 10). An additional potential cause of exaggerated airway narrowing is adaptation of ASM to shortened length. The ability of ASM to generate force decreases when it is acutely shortened (11, 12). However, when a sufficient period of time is allowed for ASM to adapt, it exhibits a remarkable ability to generate the same maximal force over a very broad length range (13, 14).

This “plasticity” or “length-adaptability” (15) of ASM has been attributed to evanescent formation or subtraction of contractile units in series and parallel (13, 16–18) and cytoskeletal remodeling (19, 20). If a similar phenomenon occurs *in vivo*, it could have a profound effect on airway mechanics during or following acute spontaneous attacks of asthma. Shortened ASM could regain its ability to generate force and shortening leading to a vicious cycle of progressive airway narrowing.

Length adaptability of ASM has been previously demonstrated by repeatedly stimulating the tissue at timed intervals under isometric conditions. This method of adaptation does not accurately represent the process of ASM adaptation that could occur in the airways of an asthmatic lung *in vivo*. For this reason, we investigated length adaptability after the shortening of porcine trachealis using a pharmacologic stimulus under conditions that mimic spontaneous *in vivo* bronchoconstriction.

Adaptation of trachealis to length change has been demonstrated previously by many independent investigators (13, 14, 16–21). This phenomenon, however, has never been unequivocally demonstrated in bronchial smooth muscle. In this study, we have included an important additional investigation showing that bronchial smooth muscle also adapts to length change and is able to regain much of its ability to generate force at short lengths.

MATERIALS AND METHODS

Tissue Preparation

Tracheas and the left lungs were removed from adult pigs immediately after the animals were killed by an overdose of sodium pentobarbital, using a procedure approved by the Animal Ethics Committee of the University of British Columbia. Tissues were stored in ice-cold carboxenated (5% CO₂ in oxygen) Krebs-Henseleit solution (details on the tissue preparation and solution composition are provided in the online supplement). Tracheal and bronchial muscle strips were prepared by dissecting away the mucosal and adventitial layers to produce a smooth muscle layer for mechanical measurements.

Equilibration Protocol and Determination of Length–Force Relationship

All muscles were “equilibrated” before experimentation. This was performed by using 12 s supra-maximal electrical-field stimulations (EFS) set to occur at 5-min intervals until a stable maximal force was achieved. The muscle was stretched to a starting length with a resting tension just enough to keep the muscle strip taut. We defined this length as reference length (L_{ref}). The L_{ref} identified in this manner was usually near the transition region between the ascending-limb and the plateau of the length–force curve determined later in the experiment. For both bronchial and tracheal strips, the force–length data points were obtained sequentially from short to long lengths. Incremental length changes were performed 60 s before stimulation. This process continued until the cumulative length increase produced a passive force with a magnitude approximately equal that of active force. At this length ($\sim 2 \times L_{ref}$), the muscle was allowed to adapt and reach its maximal potential for force generation. After full adaptation at this long length, the muscle was released to a length near L_{ref} and again allowed to adapt fully at that length.

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Simulation of Asthmatic Bronchoconstriction

The protocols used to simulate asthmatic bronchoconstriction are shown in Figures 1A and 1B. Six paired trachealis preparations were used. For each pair one preparation was used to determine changes in force (Figure 1A), while the other was used for measuring changes in shortening and shortening velocity (Figure 1B). As depicted in Figure 1A, maximal isometric force was obtained after the preparation had been adapted to its reference length (L_{ref}) (at time a, Figure 1A). Next, we shortened the muscle in the relaxed state to $0.5 L_{ref}$; this was then followed immediately by electric field stimulation of the muscle. The isometric force thus obtained (at time b) was submaximal because the muscle was not length adapted. This nonadapted (or not fully recovered) isometric force was used as a control (or baseline) to determine the degree of force recovery (measured later in this simulation protocol)

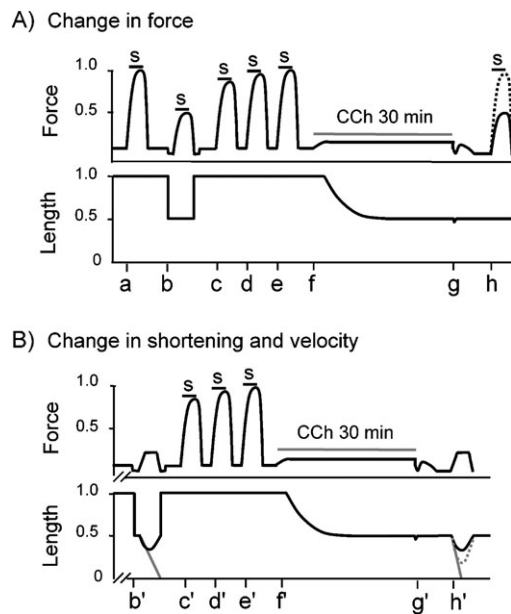


Figure 1. Asthma simulation protocol represented by time-lines (not to scale) for force and length of trachealis strips (six pairs) in response to a range of interventions. Tissues were paired, with one used to measure change in force (A), and the other for measuring change in velocity and amount of shortening (B). The letters below the time-line of A denote the following interventions: a, tissue adapted to L_{ref} ("s" and bar denote electrical field stimulation [EFS] and stimulation duration). b, tissue length decreased to $0.5 L_{ref}$ and immediately stimulated. c, d, and e, recovery of force in a three-cycle EFS-adaptation protocol at L_{ref} . f, tissues were contracted to CCh (300 nM) and shortened against a load to permit shortening from L_{ref} to $\sim 0.5 L_{ref}$ for 30 min (intervention simulates adaptation of the muscle to a short length before the final asthma attack). g, bathing solution was changed three times to remove CCh from the tissue. (Note: after the first wash, the load was temporarily reduced to zero to prevent the tissue from being stretched). h, after an additional ~ 7 min of being at $0.5 L_{ref}$ the tissue was stimulated (EFS) at the same length to simulate an asthma attack. An increase in active force (F_A) above the response observed for intervention "b", as indicated by the broken force curve, would be interpreted as being the result of length adaptation leading to excessive shortening of the tissue. The time events indicated in B are as follows: b', shortening of the tissue against a load 50% of F_A at $0.5 L_{ref}$ (the slope of the gray line indicates velocity of shortening); the broken time-line before intervention b' indicates that interventions "a" to "e" have been performed. c, d, e, f, and g are the same as in A. h', repeat of intervention "b'." An increased shortening (indicated by the broken length curve) would be interpreted as being the result of length adaptation that could be responsible for the greater airway narrowing observed in asthma attack.

after length adaptation at the same shortened length. At times c, d, and e, the preparation was brought back to its reference length and fully adapted (as seen by the full recovery of isometric force). At time point f, the muscle was stimulated by a low dose of carbachol (CCh, 300 nM), and the isotonic load was adjusted so that the muscle shortened to half of its original length ($0.5 L_{ref}$); the muscle was then allowed to remain at this length for 30 min. At the end of the 30 min (at time g), CCh was removed while the preparation was kept at the same shortened length (by varying the isotonic load). At time h, the muscle was stimulated and the isometric force obtained was compared with the control obtained at time b. Note that the stimulus and muscle length were the same at times b and h, the difference was in the history of length adaptation.

The protocol described in Figure 1B is exactly the same as that described in Figure 1A, except that at time b' and h' isotonic shortenings were induced (instead of isometric contractions in Figure 1A). An isotonic load equivalent to half of the isometric force obtained at time b (Figure 1A) was used for the isotonic contraction at times b' and h' (Figure 1B). The rate and amount of shortening measured at time h' was compared with those measured at time b'. Note that the stimulus, load, and initial muscle length at times b' and h' were all the same; the difference again was in the history of length adaptation.

Data Analysis

Curve fitting, statistics, and other data analyses can be found in the online supplement.

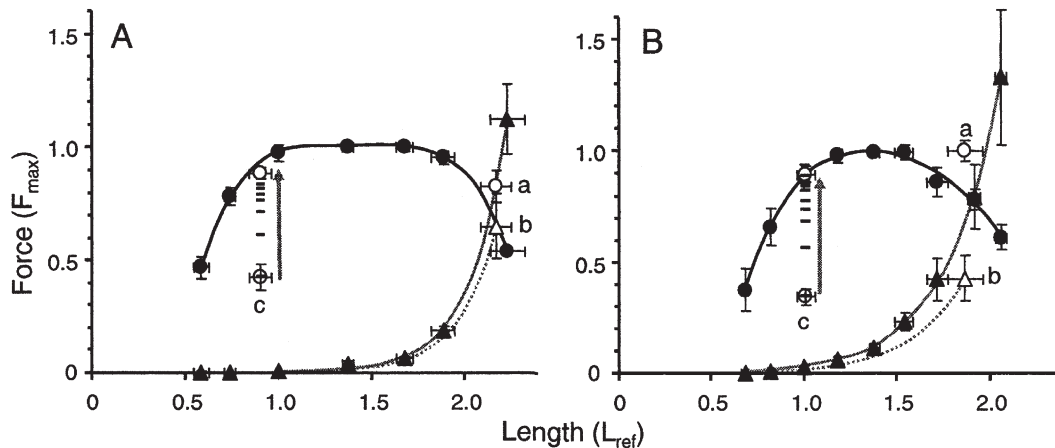
RESULTS

Figure 2 shows active and passive length–force relations of (A) tracheal and (B) bronchial smooth muscle preparations. As described in MATERIALS AND METHODS, muscle force was measured sequentially from short to long lengths with relatively small increments in length and with 5 min in between measurements. This protocol allowed some degree of length adaptation to occur in the muscle during measurement. This, however, is a common protocol in many published studies (for examples *see* Refs. 11 and 12). Both tissues exhibited a relatively broad active force plateau (solid circles), even with the incomplete adaptation at each length. After the initial determination of the length–force relationship, the muscles were allowed to adapt fully at a length close to $2 \times L_{ref}$. The maximal active isometric force (open circles) produced by both tracheal and bronchial preparations at $\sim 2 \times L_{ref}$ were labeled "a." The fully adapted active force of each preparation was higher than the partially adapted active force indicated by the length–force curve (filled circles and solid curve). The fully adapted passive force (open triangles), on the other hand, was lower than the partially adapted passive force indicated by the length–force curve (filled triangles and solid curve). The dotted lines represent projected passive length–force relationship after full-length adaptation.

After full adaptation at $2 \times L_{ref}$ (with maximal isometric force indicated by "a"), muscle preparations were acutely shortened to L_{ref} and immediately stimulated to obtain isometric force at L_{ref} without adaptation. As a result, a large decrease in active force was found (from "a" to "c"). Upon adaptation at L_{ref} , active forces of tracheal and bronchial preparations recovered substantially (with magnitude and direction indicated by the arrows). The short dashes beside the arrows indicate the levels of isometric force following each stimulation. The final adapted force at L_{ref} was not different from that at $2 \times L_{ref}$, for both preparations.

The time course of force recovery was very similar in both preparations (Figure 3), suggesting that the underlying mechanism of recovery (length adaptation) is likely the same in tracheal and bronchial smooth muscles.

Figure 4 shows the ratio of isometric force obtained at time point h over that at time point b in Figure 1A, the ratio of the



the relaxed state) to a length near L_{ref} . Subsequent to the shortening, active force decreased from that indicated by "a" to that indicated by "c." Adaptation of the muscles at the shortened length resulted in a substantial recovery of force (direction and magnitude of force recovery are indicated by the arrows). The short dashes besides the arrows indicate the level of force after each EFS during adaptation (see text for more description).

amount of isotonic shortening obtained at h' over that at b' in Figure 1B, and the ratio of the rate of shortening measured at h' over that at b' in Figure 1B. The results indicated substantial increase in the abilities to generate force and shortening after 30 min of submaximal active shortening. The increases in force, amount of shortening, and velocity of shortening were 1.93 ± 0.08 -, 1.57 ± 0.12 -, and 1.75 ± 0.2 -fold, respectively. Note that the absolute load against which the muscle shortened was the same before and after the length adaptation. Because the actively developed force was increased after adaptation, the increases in the amount and velocity of shortening are likely the result of a reduced relative load.

DISCUSSION

We have demonstrated in this study that both tracheal and bronchial smooth muscles are able to adapt to changes in muscle length and maintain their ability to generate maximal force over a large length range. This is the first time that the phenomenon

of length adaptation has been documented for bronchial preparations. (Note that we have reported this phenomenon previously [22] along with a similar report from Stephens and co-workers [23] in the form of abstracts.) Previous studies of ASM length adaptability were all done using tracheal smooth muscle (13, 14, 16–21). These results thus allow us to use the model of length adaptation developed from tracheal smooth muscle to describe the similar behavior observed in bronchial smooth muscle. Since we have hypothesized that excessive airway narrowing in asthma is, at least partially, a consequence of length adaptation, it is important that we have established that length adaptation can occur in bronchial smooth muscle. The reason we most often use the trachealis preparation is that the muscle bundle contained relatively little connective tissue and therefore very little passive force is elicited when the bundle is stretched. This has allowed us to study the mechanical properties of smooth muscle cells with little "contamination" from the nonmuscle components of the preparation.

The major finding of this study is that ASM is able to adapt

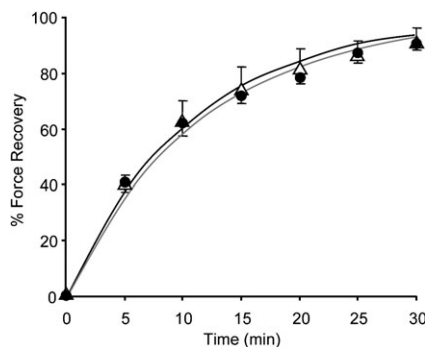


Figure 3. Time course of active force recovery (as percent of maximal recovery) for porcine tracheal (open triangles) and bronchial (filled circles) smooth muscle preparations during adaptation at $\sim L_{ref}$. The same data, in different forms, were also plotted in Figure 2 (short dashes by the arrows). The solid lines indicate the curve-fits to all data points in each group using an exponential equation and a maximum recovery of 100% for tracheal (black line) and bronchial (gray line) tissues. The two groups of data are not statistically (ANOVA) different.

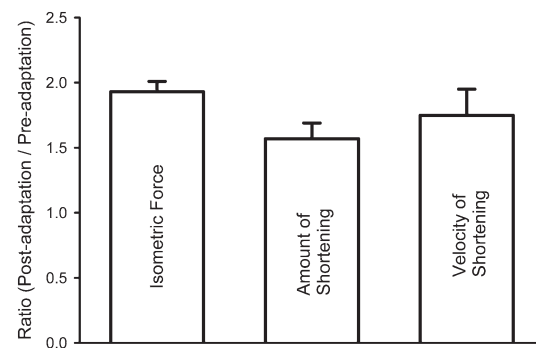


Figure 4. Ratios of post- versus pre-adaptation values of isometric force, amount of shortening, and velocity of shortening. The force ratio was obtained from measurements made at time point h (Figure 1A) divided by measurements made at time point b . The shortening and velocity ratios were obtained from measurements made at h' (Figure 1B) divided by those at b' .

and regain its ability to generate maximal force and shortening after a period during which the muscle is submaximally activated and shortened. One important difference between the present finding and previous findings from studies of ASM length adaptation is that the present protocols include adaptation of the muscle using continuous stimulation by a contractile agonist (submaximal dose of carbachol). We believe that this protocol is a more accurate simulation of the *in vivo* environment of asthmatic airways, where smooth muscle is probably chronically exposed to submaximal levels of contractile agonists that initially may cause limited shortening but with progressive length adaptation, a subsequent additional stimulus is capable of causing enhanced shortening and narrowing of the airways, contributing to the asthmatic attack.

The increase in force due to length adaptation was associated with increases in both the amount and rate of shortening, against a constant load (Figure 4). Since airway narrowing seen in asthma is closely linked to muscle shortening, the demonstration that the amount of shortening can be augmented through length adaptation allows us to interpret the present results directly in terms of airway narrowing.

The ability of ASM to adapt continuously while shortening (or in a statically shortened state) and regain its contractility, as demonstrated for the first time in this study, puts airways in a precarious state (in terms of their ability to maintain patency) when inflammatory mediators are present. There are many *in vivo* conditions under which ASM could adapt at abnormally short lengths (10, 24, 25). Chronic stimulation by inflammatory mediators could lead to muscle shortening over a prolonged period of time during which adaptation can occur. Remodeling of the airways as a result of chronic airway inflammation, which occurs in asthma and COPD, may also uncouple ASM cells from the periodic stretches imposed by tidal breathing and deep inspirations that have been shown to have bronchodilating effects (26–35). The decoupled ASM cells could adapt to a shortened length. A similar mechanism could be responsible for the airway hyperresponsiveness associated with sleep and paraplegia. During recumbent sleep, lung volume decreases and the unloaded ASM could adapt to the shortened length; such a mechanism could be one of the contributors to the characteristic exacerbation of airway narrowing that occurs during sleep in asthma. A similar mechanism could contribute to the airway hyperresponsiveness that occurs after high spinal cord injury that lowers functional residual capacity and prevents full lung inflation (36).

In summary, these data suggest that length adaptation can alter the capacity of ASM to shorten. Adaptation to short length is one mechanism that could contribute to the exaggerated airway narrowing that is a feature of inflammatory airway diseases. A corollary of these results is the possibility that adaptation to long lengths could provide a beneficial bronchoprotective effect.

Conflict of Interest Statement: B.E.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. R.R.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.D.P. is the principal investigator of a project funded by GSK to develop CT based algorithms to quantitate emphysema and airway disease in COPD. With collaborators, he has received ~\$300,000 to develop and validate these techniques. The funds he has applied solely to the research to support programmes and technicians. He is also PI of a Merck Frosst supported research program to investigate gene expression in the lungs of patients who have COPD. He and collaborators have received ~\$100,000 for this project. These funds have supported the technical personnel and expendables involved in the project. C.Y.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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METHODS

Muscle preparation and experiment apparatus

Bundles of tracheal and bronchial muscles were dissected under a binocular dissecting microscope. The average dimension of the muscle strips were ~6 mm long, ~1.5 mm wide and ~0.3 mm deep. The tissue strips were connected to aluminum foil clips and mounted vertically in a muscle bath with the lower end fixed to a stationary hook and the top end attached to a servo-controlled lever of a length-force transducer. The muscle bath contained Krebs-Henseleit solution (see below) at 37°C, pH 7.4 and gassed with 5% CO₂ in oxygen.

The length-force transducer was computer controlled and was capable of measuring force while holding the muscle length constant (isometric mode). It was also able to measure muscle shortening under a constant load (isotonic mode). The computer also controlled the onset and termination of electrical field stimulation (EFS) by turning on and off the stimulator at pre-determined times. The current-boosted stimulator delivered supramaximal stimulation (60 Hz sinusoidal wave) via two platinum electrodes to the muscle at 20 V (peak-to-peak). The apparatus has been described previously (E1).

Physiological saline solution

Krebs-Henseleit solution contained (in mM): 118 NaCl, 5 KCl, 2 CaCl₂, 2 MgSO₄, 1.2 NaH₂PO₄, 22.5 NaHCO₃, 11.1 D-glucose, (Sigma Chemicals, St. Louis, MO).

Adaptation of muscle preparations at a reference length

The adaptation protocol was performed using 12-second supra-maximal electrical-field stimulations (EFS, 60Hz alternating current, set at 20V), which were set to occur at 5-minute

intervals. Initial adaptation was performed at a length with a resting tension just enough to keep the muscle strip taut but without much stretching of the tissue. We defined this initial length as reference length (L_{ref}). The reference length identified in this manner was found to be usually near the transition region between the ascending-limb and the plateau of the length-force curve determined later in the experiment. In some experiments, however, the initial length was set too short. The length had to be corrected with the following protocol: if the active force generated at the initial length after adaptation was less than 70% of the maximal (plateau) force generated during the length-force (L-F) protocol (described below), tissue length was increased by 20% and re-adapted at the new length before determining a new L-F relationship. This procedure was repeated until a “correct” L_{ref} was determined. All measurements of force were determined under isometric conditions.

Initial determination of length-force relationship

During the equilibration period, the force generated at L_{ref} was monitored. When the active force reached a steady plateau, the protocol for establishing the L-F relationship was then carried out. For bronchial strips, the first force data point was obtained at $0.68 L_{\text{ref}}$ and the mean lengths to be set sequentially for force measurements were: 0.82, 1.0, 1.18, 1.38, 1.54, 1.72, 1.92 and $2.06 L_{\text{ref}}$. Increments in length were stopped at the length where passive force was greater than active force. The mean maximal length used was $1.88 \pm 0.10 L_{\text{ref}}$ for the bronchial preparations. Length changes were performed 60 seconds prior to stimulation. A similar protocol was used for tracheal strips except that greater length increments were used. Starting length was $0.59 L_{\text{ref}}$ and the other lengths to follow were: 0.74, 1.0, 1.37, 1.68, 1.89 and $2.24 L_{\text{ref}}$. The mean maximal length used for the tracheal preparations was $2.24 \pm 0.10 L_{\text{ref}}$.

Control observation: Force increase due to length adaptation is independent of activation mode

The tissues that were used for the asthmatic-bronchoconstriction-simulation experiments were adapted to a short length in response to carbachol-activation for 30 minutes. The increase in force measured at $0.5 L_{\text{ref}}$, which we interpreted as evidence for length adaptation, could alternatively be explained based on CCh causing up-regulation of biochemical factors (independent of length) involved in force generation. For example, increased agonist-receptor coupling or increased calcium flux, and not length-adaptation as suggested by our previous studies (E2), could be responsible for our observation. It was important, therefore, to demonstrate that CCh activation alone could not produce the observed increase in force after the length adaptation protocol. For these control experiments, tissues ($n=3$ pigs) were initially adapted at L_{ref} by using the EFS protocol. After adapting tissues at L_{ref} , tissue length was decreased to $0.5 L_{\text{ref}}$ followed by EFS 15 seconds later. This force measured without adaptation at $0.5 L_{\text{ref}}$ served as a control measurement. Tissues were then re-lengthened to L_{ref} in the absence of active muscle tone and were re-adapted using the EFS protocol. After re-adaptation, the tissues were activated by CCh (300 nM) at L_{ref} for 30 minutes and then allowed to recover for 7 minutes after removal of CCh; the same recovery time was used for the asthmatic-bronchoconstriction-simulation protocol (described in main text). Following the recovery period, muscle length was decreased to $0.5 L_{\text{ref}}$ and 15 seconds later the muscle was activated by EFS. To complete the control study tissues were re-lengthened to L_{ref} and adapted using the EFS protocol for 30 minutes. Figure E1 summarizes the results. Active force (F_A) in response to EFS obtained at $0.5 L_{\text{ref}}$ (0.31 ± 0.05 , F/F_{max}), measured immediately after releasing the pre-contracted tissues (CCh, 300 nM, 30 minutes) from L_{ref} , were not different from control F_A EFS values

obtained at 0.5 L_{ref} without adaptation (0.40 ± 0.09 , F/F_{max} , $P > 0.05$, $n=3$ pigs). The relaxed control tissues were manually shortened from L_{ref} to 0.5 L_{ref} and followed by EFS stimulation. This result indicated that pre-treatment of tissues with a cholinergic agonist did not up-regulate (or prime) subsequent EFS responses at 0.5 L_{ref} .

Data Analysis and curve fitting

Active and passive forces were normalized to maximum active force developed at L_{ref} (F_{Amax}). Active force (F_{A}) data were fitted by eye. Passive force (F_{P}) data were fitted to an equation for exponential growth: $F_{\text{P}} = a \cdot (\exp(b \cdot x) - 1) / b$ where a and b are constants and x is the length normalized to L_{ref} . The equation that gave the least mean squares difference between actual and predicted values was used for the fitted expression. Force recovery data were fitted to single 2- or 3-parameter exponential-rise-to-maximum equations; $F_{\text{A}} \text{ recovery} = y_0 + a \cdot (1 - \exp(-b \cdot t))$, where y_0 , a and b are constants, and t is the time from the first force measurement. Microsoft[®] Excel 2002 was used for data management, SigmaPlot[®] 8.0 for plotting and fitting graphs and Statview[®] 5.01 for performing statistical analyses. Factorial analysis of variance (ANOVA) and Fisher's protected least-squares difference test were used to detect differences ($P < 0.05$) in the time course of force recovery (program JMP 4.0.4).

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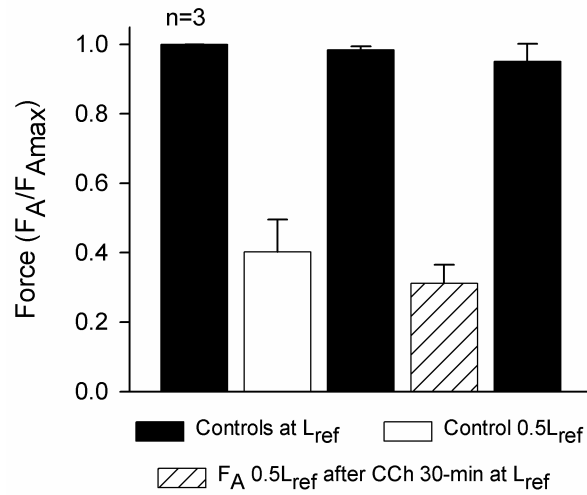


Figure E1. Control observation that demonstrates tissue viability after carbachol (CCh) treatment at L_{ref} for 30 minutes and the effect on the subsequent responses at 0.5 L_{ref} . Column order is in chronological sequence. There was no significant reduction in F_A (responses to EFS) at L_{ref} over the entire experiment period (~2 hrs, $P > 0.05$, filled columns). Control F_A at 0.5 L_{ref} (empty column, no CCh treatment) was significantly less than F_A at L_{ref} ($P < 0.0001$). The test F_A at 0.5 L_{ref} (hatched column, obtained after treatment of CCh (300 nM) at L_{ref} for 30 minutes) was not significantly different from the control (empty column).