THE ENERGETICS OF SMOOTH MUSCLE CONTRACTION IN ALLERGIC BRONCHOSPASM

BY

YIQING MA

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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Abstract

For various questions concerning the kinetics of the actin-myosin-interaction in muscle or muscle regulation, it is desirable to measure mechanical, energetics and optical properties simultaneously using normal and skinned muscle fibers.

In the present study NADH fluorescence method was used on skinned canine tracheal smooth muscle to elucidate: 1) the energetics of allergic bronchospasm; and 2) the validity of the plasticity theory of smooth muscle contraction. The results showed an increase in the maximum shortening velocity and maximum shortening capacity in the skinned tracheal smooth muscle from a ragweed pollen-sensitized canine model. Comparing the result of the intact muscles, we concluded that both changes in the contractile apparatus and the muscle cell membrane contributed to the increased mechanical properties 2) length dependent ATP consumption rate in smooth muscle, which provided evidence of the plasticity theory in such muscle.

Future work will be focused on two directions: one will be the comparison of the message levels of MLCK content between sensitized and control groups so as to account for the increased protein content of MLCK. The other direction will be an investigation of the reasons for the increased myosin heavy chain ATPase activity which includes the measurement of contents of certain proteins that influence the myosin heavy chain ATPase activity. Both of studies will contribute to further elucidation of the mechanism of allergic bronchospasm.

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2. To elucidate by bioenergetic studies the validity of the plasticity theory of smooth muscle contraction.

Chapter I: Mechanical alterations of skinned tracheal smooth muscle in a ragweed pollen-sensitized canine model

Chapter II: ATP utilization in cross-bridge cycling of skinned airway smooth muscle from ragweed pollen-sensitized dogs and littermate controls

Objective 1:

To delineate the mechanical alterations that develop in activated skinned sensitized tracheal smooth muscle and from the concomitant studies of energy utilization to elucidate the reasons for the mechanical alterations in canine sensitized tracheal smooth muscle.

Chapter III: Length dependent ATP consumption rate in smooth muscle: evidence for plasticity

Objective 2:

To study the alteration in energy utilization rate that occurs with length change and thus to provide support for the recently described plasticity theory of muscle contraction in canine airway smooth muscle.

Chapter I: Mechanical alterations of skinned tracheal smooth muscle in a ragweed pollen-sensitized canine model

Abstract

For a long time people have thought that the increased airway narrowing in asthmatics was solely because of increased neural activity. However, we have recently reported that in sensitized tracheal smooth muscle, the maximal shortening velocity (Vo) and the maximal shortening capacity (ΔLmax) were increased, which demonstrated that the alterations in contractility were primarily stemming from the muscle itself. Furthermore, Dr. S. Sigurdsson, a collaborator, using the whole cell patch clamp technique, found decrease in the delayed rectifier current (I^{+}_{DR}) which would account for increased sensitivity of the sensitized cell. In the present study, we measured the mechanical properties of skinned tracheal smooth muscle (TSM) from sensitized and control dogs by using the Güth muscle lever system. We found no change in maximum active tension (Po), but Vo and ΔLmax in sensitized TSM increased by 24.63±3.69(SE)% and 14.12±1.74(SE)% respectively. Hence both the Vo and ΔLmax of skinned sensitized TSM were significantly greater than those of the controls ($p<0.001$) but the level of increase was less than that shown in the intact muscle. Furthermore, our calculations allowed us to conclude 69.44% of the increased Vo resulted from changes in the contractile apparatus of the smooth muscle and the remaining 30.56% from the membrane. Both of them permit increased shortening velocity which may account for allergic bronchospasm. ATP-γ-s is believed to be a substrate for myosin light chain kinase but not for actin-activated myosin ATPase and can be used to thiophosphorylate the myosin light chain of the chemically skinned smooth muscle preparation. Thiophosphorylated myosin is a poor substrate for myosin light chain phosphatase, leading to essentially irreversible phosphorylation of myosin, which is therefore independent of Ca^{++}. In the present study the mechanical properties between with and without ATP-γ-s treated skinned preparations were also compared to study the effect of

ATP-γ-s on Po and Vo which can elucidate further the role of myosin light chain phosphorylation. We found that both Po and Vo showed no difference between the two thiophosphorylated groups ($p>0.05$), which suggested that Po and Vo were not related to the level of myosin light chain 20 (MLC_{20}) phosphorylation in a simple fashion. We concluded that after a certain level of MLC_{20} phosphorylation, these parameters will not be further dependent on MLC_{20} phosphorylation. It must be pointed out, however, that in human and canine asthma maximal levels of phosphorylation are not found. Perhaps at the lower *in vivo* levels differences in these mechanical parameters could be present.

Literature Review and Background

Although the pathophysiological changes leading to asthma are not yet well known, increased airway responsiveness to a number of allergic and nonallergic stimuli is an important aspect of the disease. One example is the dose-response histamine-inhalation test. Histamine aerosol inhaled in small doses is thought to act chiefly as an irritant and stimulate vagal parasympathetic reflex bronchoconstriction (Cockcroft et al. 1977). Histamine may also be acting through a direct effect on bronchial muscle. Non-specific bronchial reactivity was increased in 3% of presumed normal human subjects, in 100% of active asthmatics and in 69% of asymptomatic asthmatics with previous symptoms only at times of exposure to clinically relevant allergens. Tracheal smooth muscle (TSM) from an ovalbumin sensitized canine model of allergic asthma also showed hypersensitivity and hyper-reactivity to histamine when compared to that from littermate controls in vitro (Antonissen et al. 1977). For a long time, these have led to considerable investigation into the sequence of events producing IgE-mediated (allergic) bronchoconstriction, with major emphasis on the role of antigen-antibody interactions in the airway, the effect of both local and systemic mediator release on the airway and the modulation of the events by both the sympathetic and parasympathetic nervous system (Antonissen et al. 1980). However, sensitized canine tracheal smooth muscle has been shown to also undergo non-specific alterations in mechanical properties (Antonissen et al.1980, Boushey et al. 1980, Stephens et al. 1988, 1985). Antonissen et al. have observed, in the sensitized TSM, an increase in the maximum velocity of shortening (Vo) and in Hill's (1938) b constant in isotonic experiments, and the development of spontaneous phasic mechanical activity and of a myogenic response in isometric studies (Antonissen et al. 1980). Along with the observation of Cockcroft et al., those findings suggest that the essential defect in allergic bronchial hyper-

responsiveness might be at muscle cell level. The role of airway smooth muscle as the effector in allergic bronchial hyperresponsiveness has recently been considerably investigated (Stephens et al. 1988, 1985). Studies in our laboratory showed that both in tracheal and bronchial smooth muscle, the mechanical properties of sensitized muscle were altered. Both the maximal shortening velocity (Vo) at 2s and the maximal shortening capacity (ΔLmax) were significantly greater than those of the controls (Stephens et al. 1991, Jiang et al. 1992). The muscle possessed a contraction time of at least 10s, however, our studies of the canine bronchial muscle have shown that, as in the hog carotid (Jiang et al. 1990), normally cycling and latch bridges are present. The former, which are active for the first 2s of contraction, cycle at a relatively fast rate and are responsible for effecting 75% of the total isotonic shortening of the muscle. As this occurs within 2s, it is obvious that velocity of shortening could be a limiting factor to shortening. The increased Vo and ΔLmax may represent a basic alteration in the airway smooth muscle contractile apparatus that contributes to the generation of bronchoconstriction in allergic asthma. Furthermore, Sigurdsson, one of our collaborators (Smooth Muscle Research Group, Faculty of Medicine, University of Calgary), studied the properties of macroscopic voltage-gated delayed rectifier K^+ current (I_{KDR}) of isolated airway smooth muscle cells from fourth to sixth order canine bronchi using the whole-cell patch clamp technique (22°C) (Waldron et al. 1997). Whole-cell voltage clamp studies indicated that macroscopic K^+ current due to KDR (I_{KDR}) is a dominant outward conductance between membrane potentials of -60 and 0 mV in tracheal myocytes of ferret, canine and humans (Kotlikoff 1990, Fleischmann et al. 1993, Adda et al. 1996). Sigurdsson found the capacitative surface area, current density at +30 mV and voltage-dependence of activation of I_{KDR} of bronchial myocytes from ragweed pollen-sensitized dogs were not different from those of age-matched control dogs.

However, the steady-state availability of I_{KDR} was reduced between -50 and -20 mV in myocytes from ragweed pollen-sensitized dogs due to a 4mV hyperpolarizing shift in $V_{0.5}$ for half-maximal inactivation compared to the value for I_{KDR} of myocytes of control dogs. The amplitude of window I_{KDR} over the physiological range of membrane potentials of canine bronchial myocytes was found to be reduced by : 1) a hyperpolarizing shift in voltage-dependence of inactivation associated with ragweed pollen-sensitization, and 2) activation of protein kinase C (PKC) which depressed the magnitude of I_{KDR} (Aiello et al. 1995, 1996, Clement-Chomienne et al. 1996, Cole et al. 1996). Enhanced PKC activity was also implicated in the pathogenesis of asthma by Baron et al. 1994, Schramm et al. 1992, Rosetti et al. 1995 and Horowitz et al. 1996. This result demonstrated that the function of the cell membrane in the allergic bronchospasm was altered.

Therefore, the question is whether bronchoconstriction originates from the contractile apparatus or the cell membrane. The present study was undertaken to determine the mechanical properties of Triton X-100 skinned tracheal smooth muscle from ragweed pollen-sensitized dogs and littermate controls. Triton X-100 skinning destroys plasmalemmal and intracellular membranes, thereby functionally extracting cell membranes and isolating the contractile machinery (Gabriele et al. 1995). Thus the skinned preparation can be a model for the contractile system of the intact muscle. By using Triton X-100 skinning we are able not only to compare mechanical properties between intact and skinned preparations but also between skinned sensitized and control tracheal smooth muscle and conclude to what extent the mechanical alterations of the intact sensitized airway smooth muscle are due to altered membrane function or contractile apparatus alterations.

ATP-γ-s is believed to be a substrate for myosin light chain kinase but not for actin-activated myosin ATPase (Wysolmerski et al. 1991) and can be used to thiophosphorylate the myosin light

chain (Hoar et al. 1979) of the chemically skinned smooth muscle preparation. Thiophosphorylated myosin is only a poor substrate for myosin light chain phosphatase (Sherry et al. 1979), leading to essentially irreversible phosphorylation of myosin, which is therefore independent of Ca^{++}. In the present study the mechanical properties between skinned preparations with and without ATP-γ-s treatment were also compared to study the effect of ATP-γ-s on Po and Vo in order to elucidate further the role of myosin light chain phosphorylation.

Methodology

In Vivo Sensitization

Newborn dogs were randomly divided into sensitized (S) and control (C) groups. The S group was sensitized according to the method described by Kepron (Kepron et al. 1977). They received intraperitoneal injections of 500 ug of ragweed pollen extract in 30 mg of an aluminum hydroxide adjuvant within 24 h of birth. The booster injections were repeated weekly for 8 weeks and at biweekly intervals thereafter. This method of immunization (Kepron et al. 1977, Pinckard et al. 1972) has been shown to induce prolonged immunoglobulin E antibody production of high titres against the ragweed pollen extract. Their littermate controls received, at the same time, injections of the adjuvant alone. Sensitization to ragweed was determined by the homologous passive cutaneous anaphylaxis test. A sensitized dog demonstrating passive cutaneous anaphylaxis titres > 1:256, and its nonsensitized littermate control were selected randomly on a given day for the *in vitro* studies. In previous *in vivo* studies (Kepron et al. 1977), all sensitized littermates with passive cutaneous anaphylaxis titres > 1:64 developed marked increases in specific airway resistance on bronchoprovocation with ragweed extract aerosol compared with controls similarly challenged. In addition, sensitized dogs were fourfold more responsive to acetylcholine than their littermate

controls (Becker et al. 1989).

In Vivo Mechanical Studies

Preparation of Muscle Strip

The trachea from an anaesthetized dog was rapidly removed and placed in a beaker of ice-cold Ca^{2+} -free Krebs-Henseleit solution, composed of (in mM); 112.6 NaCl, 25 $NaHCO_3$, 1.38 NaH_2PO_4, 4.7 KCL, 2.46 $MgSO_4$ and 5.56 dextrose, aerated with 95% O_2 and 5% CO_2 and a strip of muscle was dissected out in gassed Ca^{2+} -free Krebs-Henseleit solution under a dissecting microscope. The strips used in such experiments possessed an average length of 4.5mm, thickness of 0.2 mm, width of 0.3 mm and dry weight of 0.07 mg. The tissues could be maintained in a viable state for at least 4 days at 4°C. In this study, to ensure maximal mechanical responses, all experiments were performed within two days of removal from the animal.

Skinning Procedure

Thin strips of TSM were detergent skinned using Triton X-100 as described by Cortijo et al (1987). The strips were exposed to a skinning solution containing (in mM) 50 K-acetate, 150 sucrose, 1 dithiothreitol (DTT), 5 ethylene glycol-bis (β-aminoethyl ether)-N, N, N, N-tetraacetic acid (EGTA), 20 Imidazole and 1% Triton X-100 (pH 7.4 at 22°C with KOH), for 2 hours at room temperature.

Mechanical Data Acquisition System

Skinned muscle strips to be used for measurement of maximal shortening capacity and velocity were mounted in a lever system designed by Güth (1986). The strips were mounted between two stainless steel tweezers, one of which was attached to a force transducer and the other to a servo-motor, in a small ($1x1mm^2$) square quartz cuvette containing high-EGTA relaxing solution for equilibration and then changing to low-EGTA relaxing solution.

The strips were stretched to a length that produced 40-60 mg resting force. This level of passive force was about 10% of maximal active force produced by this smooth muscle preparation. If the passive force of intact or Triton X-100 detergent-skinned canine tracheal smooth muscle is 10% of the average maximal active force, the strips are near the length at which maximal active force is developed, defined as Lo (Herlihy et al. 1973, Morland et al. 1988). The mounted strips were perfused with fresh, low- EGTA relaxing solution changed every 20 seconds until a stable resting force was obtained. Contractions were initiated by introducing the contracting solution, which has the same composition as low-EGTA solution, except for the inclusion of 25μM free Ca^{2+}. Total ion concentrations were calculated from the desired free ion concentrations by a computer program based on the multiple ionic equilibrium equations using binding constants.

Isometric Measurements

The muscle strips were allowed to contract isometrically at the optimal length (Lo) at which maximum force (Po) (see Figure 1) is elicited. Force values were converted to stress and were expressed in milli-Newtons force per square millimeter cross-sectional area of muscle. The latter was estimated using the length of the tissue and its dry weight, while assuming the tissue to have a specific gravity of unity and a cylindrical shape (Stephens et al. 1992). The details are described in *Morphological Measurements* below.

Isotonic Measurements

When the muscle strips were stable with respect to the maximal active force developed isometrically, they were quickly released to an isotonic load (5% of the maximal force), which was lighter than the maximal active force that the muscle strips developed; this allowed the strips to shorten to the maximal extent (see Figure 1). This resulted in a rapid transient due to shortening

of the muscle's series elastic component (SEC) followed by artifactual oscillations and then a slow transient. The maximal slope of the slow transient (the maximum shortening velocity Vo) was computed and identified as the maximal shortening velocity of the contractile element (CE) (see Figure 1). Vo was expressed in optimal muscle length per second (Lo/s).

The maximal shortening capacity (ΔLmax) of the muscle strips was directly measured from the isotonic shortening traces from the quick release experiments (see Figure 1). The ΔLmax was expressed in per optimal muscle length (/Lo).

Thiophosphorylation of Myosin Light Chain 20 (MLC_{20}) with ATP-γ-s

The above experiments were conducted in one group of muscle strips after exposure to 1mM ATP-γ-s for 30 minutes in the presence of 25uM Ca^{++}. MLC_{20} was thiophosphorylated and after which contraction became independent of Ca^{++}.

Morphological Measurements

Muscle length was measured using an OLYMPUS stereo microscope attached to the lever system. An autobalance was used to determine the muscle dry weight.

Statistical Analysis

All values were given as means ± SE with the number of observations within parentheses. The mean values of Po, Vo, ΔLmax and the values of standard error were calculated by theSigmaPlot program. The mean values from sensitized and control groups were compared using an unpaired two-tailed t test with the probability, p set at a maximum of 0.05 to determine significance.

Results

Isometric Studies

The maximum isometric force of the two groups are shown in Figure 2 and Table 1. When

ATP-γ-s was not used, the maximum force of control and sensitized groups were 170.45±7.06mN/mm^2 and 179.79±24.65mN/mm^2 respectively. When ATP-γ-s was employed, they were 143.16±12.54mN/mm^2 and 150.04±4.84mN/mm^2. Whether in the presence or absence of ATP-γ-s, there was no significant difference of Po ($p>0.05$) when normalized with respect to muscle cross-sectional area between control and sensitized groups. As well no significant difference was found between the groups treated with and without ATP-γ-s ($p>0.05$). Furthermore, the Po of sensitized and control groups in skinned preparations was higher than what we had found in the intact group (sensitized: 145.24mN/mm^2; control: 147mN/mm^2) (Stephens et al. 1991).

Isotonic Studies

Maximal shortening velocity. Figure 3 and Table 2, display the Vo of sensitized and control skinned tracheal smooth muscle. They were 38.85±1.15 (%Lo/sec) and 31.17±1.24 (%Lo/sec) respectively in the without ATP-γ-s treated group. A highly significant difference was found between the two groups ($p<0.001$). The Vo of the sensitized group was significantly increased by 24.63±3.69% compared to the control; it was also lower than that found in the intact muscle (35.47%). When treated with ATP-γ-s, the Vo's were 39.79±1.74 (%Lo/sec) and 33.67±0.83 (%Lo/sec) respectively. The difference between them was significant($p<0.05$). The Vo increased by 18.17±5.18% in the sensitized group. However, no significant difference was found between the with and without ATP-γ-s treated groups ($p>0.05$). In a previous study we have reported that the Vos measured at 3s of sensitized and control groups in intact preparations were 0.401 and 0.296 (%Lo/sec) at 37°C. We have also reported that the Q_{10} value of Vo for trachealis was 1.8 (Stephens et al. 1977). With this information we were able to calculate that the Vo of STSM and CTSM were

0.149 and 0.110 (%Lo/sec) at 22°C which were lower than those we found in the skinned preparations ($p<0.05$).

Maximum Shortening Capacity. The values of ΔLmax of all groups are displayed in Figure 3 and Table 2. Significant differences ($p<0.05$) were found between muscle strips from sensitized dogs and their littermate controls in both ATP-γ-s treated and untreated groups. In the absence of ATP-γ-s, the ΔLmax of sensitized dogs was 88.72±1.35(%Lo) and 77.74±0.73(%Lo) in the control dogs. Hence, in sensitized dogs, it increased by 14.12±1.74%. In the presence of ATP-γ-s, ΔLmax were 85.16±1.36 (%Lo) and 77.80±1.57 (%Lo) respectively. An increase of 9.46±1.74% was found in the sensitized group. There is no significant difference ($p>0.05$) between groups with and without ATP-γ-s treatment. We have reported in intact preparations that the ΔLmax values of STSM and CTSM were 64.3 and 53.1 (%Lo) respectively which were significantly lower than those we obtained from the skinned muscle.

Discussion

Isometric Studies

Compared to skinned control tracheal smooth muscle, the Po in skinned sensitized group was unchanged, indicating that isometric parameters are insensitive indicators of disease. This result is consistent with what we obtained from the intact muscle. Isometric properties of airway smooth muscle relate to the stiffness of the bronchial wall only, and their role in regulating resistance is minor. Had purely isometric studies been conducted one would have concluded the sensitized muscle was not different from unsensitized muscle. However a clear cut difference in maximum shortening is evident. This points out the importance of isotonic studies in detecting early disease. Also, isotonic shortening is the appropriate parameter to study, as it is the *in vitro* analogue to *in vivo*

bronchoconstriction. Furthermore inasmuch as the resistance to flow in a tube depends on its diameter, flow regulation in airways or blood vessels, which are directly controlled by shortening or elongating rather than stiffening of smooth muscle, is best studied *in vitro* by evaluation of isotonic parameters such as the Vo and ΔLmax (Stephens et al. 1988). In this study, the Po between groups with and without ATP-γ-s treatment also showed no difference. In previous work (Sparrow et al. 1981, Ruegg et al. 1982), it has been shown that the level of isometric force is dependent on both the Ca^{++} and calmodulin concentrations. These results suggested that the number of activated cross-bridges are dependent on the activity of myosin light kinase, a Ca^{++}-calmodulin-dependent enzyme. ATP-γ-s is a substrate for myosin light chain kinase, but thiophosphorylated myosin is only a poor substrate for the phosphatase (Sherry et al. 1978). Thus the use of ATP-γ-s leads essentially to irreversible phosphorylation of myosin, which is thereafter independent of $[Ca^{++}]_i$ (Cassidy et al. 1979). In the presence of ATP-γ-s, the level of thiophosphorylated myosin light chains in skinned fibres is reported to be substantially higher than the levels of phosphorylation achieved at saturating Ca^{++} concentrations (Paul et al. 1983) in the absence of ATP-γ-s. Both skinned taenia coli (Sparrow et al. 1981) and carotid artery (Peterson 1980) smooth muscles develop maximal isomeric force which is independent of Ca^{++} after exposure to ATP-γ-s. However it is also known that near maximal stress is generated with only 35% of the cross-bridges in the phosphorylated state in intact preparations (Berne et al. 1990). In skinned preparations Kenney also demonstrated that the relationship between tension and light chain phosphorylation was linear and with a steep gradient when myosin light chain phosphorylation was below 20%. Once approximately 20% of the total number of light chains were phosphorylated, the fibre bundles generated maximum force (Kenney et al. 1990). In this study we obtained 80% and 100% phosphorylation levels in groups treated with

and without ATP-γ-s respectively. At these high phosphorylation levels no difference in Po from two groups was detectible. When we compared the skinned and intact preparation, we found the Po of the skinned group was larger than that of the intact group. This is different from what Arner (1982) and Peterson (1982) found. They found lower active force in skinned preparations as compared to that in the maximally activated intact muscle. The Po values we obtained in skinned preparations in this study were also higher than those reported by Peterson (100mN/mm^2; 1982), Iino (28-60mN/mm^2; 1981) and Arner (67-100mN/mm^2; 1982). The reasons for the above results might be 1) the higher level of MLC_{20} phosphorylation we found in the skinned preparations (≥80%) compared to what we found in the intact preparations (26.3%) (Jiang et al. 1995) and 2) the weight of the muscle we measured in this study was the dry weight which in the force normalization process would result in a decreased cross sectional area and cause the high value of Po while the weights of intact muscle used in our other studies were wet weights. 3) since no comparison of MLC_{20} phosphorylation was conducted between skinned and intact groups by Arner and Peterson, it is still difficult to explain the main reason why we obtained the different results.

Isotonic Studies

In this study we found an increase of 24.63% in maximum shortening velocity in skinned STSM without ATP-γ-s compared to CTSM. This could be because of the increased myosin light chain kinase content in STSM we have reported, which results in increased myosin light chain 20 (MLC_{20}) phosphorylation (Wang et al. 1983). It can also be explained by the increased myosin heavy chain ATPase activity (see Chapter II). Using ATP-γ-s, we still found an increase of 18.17% in the maximum shortening velocity in STSM. Because ATP-γ-s can fully thiophosphorylate MLC_{20}, 100% phosphorylation was obtained in both sensitized and control groups; therefore this increase

of Vo thus can not be explained by increased MLC_{20} phosphorylation. However it is consistent with increased actin-activated ATPase activity in the STSM which we obtained by using ATP-γ-s in the energetics studies. We still do not know the reason for the increased actin-activated ATPase activity, but the distribution of myosin heavy chain isoforms, the proportion of myosin light chain 17 isoforms, the content of calponin, caldesmon, tropomyosin, and a myosin heavy chain isozyme with a seven amino acid insert (sm-B) in its NH_3-terminal end may all play roles in this result .

In the intact preparations, the Vo for the 2-s of contraction in the sensitized tracheal smooth muscle was 0.401Lo/s, while in control group it was 0.296Lo/s. Therefore the Vo for the 2-s of sensitized group increased by 35.47% (Stephens 1991), which is greater than what we found in the skinned preparations (24.63%). Since we used Triton X-100 skinning in the present study, and Triton X-100 skinning can destroy the cell membrane leaving behind the contractile proteins, the difference between the two increased levels of Vo from intact and skinned preparations should be due to the changes in muscle cell membrane function. Sigurdsson, using the whole cell patch clamp technique, found decrease in the delayed rectifier current (I^+_{DR}) which would account for increased sensitivity and reactivity of the sensitized cell (Gareth et al. 1997). This result demonstrated the function of the cell membrane in allergic bronchospasm, while the finding in the skinned preparation may represent a basic alteration in airway smooth muscle contractile apparatus that contributes to the generation of bronchoconstriction in allergic asthma. This allowed us to compare data between intact and skinned preparations and to determine that 69.44% of the increased Vo comes from the contractile apparatus and the remaining 30.56% from the membrane. Thus, both parts of the muscle cell contribute to increased shortening velocity and account for the hyperreactivity of the ragweed pollen-sensitized tracheal smooth muscle.

When we compared the Vo between the groups treated with and the without ATP-γ-s, no significant difference was found, which is consistent with the result of Paul (1983). Paul demonstrated that for both skinned taenia coli and carotid artery, Vo was dependent on the Ca^{++} and calmodulin concentrations. Arner 1983 reported Ca^{++} dependence of Vo in skinned portal vein. These findings indicate that Vo is related to the level of myosin light chain phosphorylation, mediated by Ca^{++}-calmodulin dependent myosin light chain kinase. This hypothesis is supported by the observations of Cassidy (1981) and Arner (1996). In the current study we found 80% and 100% of phosphorylation levels in the two groups with and without ATP-γ-s treatment but no change in Vo. The reasons could be 1) after a certain high level of MLC_{20} phosphorylation is achieved, Vo reaches a saturating level and is not dependent on phosphorylation any longer, and 2) other regulatory mechanisms may play roles in determining Vo.

Arner (1982) found Vo was unaltered in the skinned preparation compared to the intact muscle indicating similar kinetics of actomyosin interaction in the presence of $10^{-4.5}$M Ca^{2+}. However in our study we found a higher Vo in the skinned preparation which may due to the higher MLC_{20} phosphorylation level ($\geq$ 80%) (see Figure 8, 9) we obtained in the skinned preparations than the 26.3% we had previously obtained in the intact muscle (Jiang et al. 1995).

An increase of 14.12% in ΔLmax in the sensitized group was found in this study. It would translate into a 83.7% increase in airflow resistance in sensitized model by using Poiseuille's equation, which applies here, because the flow in this portion of the respiratory tree is laminar, (assuming that all other variables were held constant). This is significant with respect to allergic bronchoconstriction. The increased shortening of sensitized airway smooth muscle could be the result of two factors. One factor is an internal resistance to shortening. The evidence that such a

phenomenon exists is straightforward. If one stretches a strip of muscle to beyond Lo and then releases it, it returns to Lo. Furthermore, if one allows a stimulated muscle to shorten maximally and then removes the stimulus the muscle re-elongates to its original length. It is as if there was an elastic resistance to shortening and stretching in the cell. In the shortening mode this resistor undergoes compression and stores potential energy. When the stimulus is turned off the resistor recoils and restores the muscle to its original length. We have demonstrated that the compliance of the internal resistance to shortening of the sensitized group was increased, which could account for the increased shortening of the muscle. What causes the increased compliance of the internal resistor in the sensitized trachealis is still unknown. While collagen and elastin and other structures in the extracellular and extra-fascicular spaces are strong contenders, the finding that the passive properties (resting tension) from both groups were the same does not support the idea that changes in collagen and elastin are responsible. At the cellular level the cytoskeleton could play a very important role in influencing resting tension. It is now recognized that the cytoskeleton is a very important component of any cell and especially of the smooth muscle cell. Furthermore it forms an exquisitely organized network around and through the sarcomeres of striated muscle (Wang et al. 1983). It has been reported that low doses of ionizing radiation of skinned skeletal muscle cells induced changes in two cytoskeletal proteins, nebulin and titin, resulting in decreased passive and active tensions in response to calcium (Horowits et al. 1986). While these experiments highlight the importance of the cytoskeleton in muscle contraction, however titin and nebulin have not been found in smooth muscle. On the other hand there are a large number of small molecular weight cytoskeletal proteins present in smooth muscle such as filamin, desmin, vimentin, vinculin, plectin, α-actinin, and synemin (Rasmussen et al. 1987), some or all of which could contribute to the

structure of the internal resistor. Rasmussen et al. 1987 have shown that these proteins are phosphorylated late in contraction. This could alter the mechanical properties of the cytoskeletal network and, therefore change the properties of the internal resistor. Another factor that contributes to the increased shortening in sensitized airway smooth muscle is the increased Vo. It was felt initially that the velocity of shortening was not an important consideration in determining the increased shortening of the airway smooth muscle. As the muscle possessed a contraction time of at least 10s, it seemed that velocity would not limit the maximal amount of developed shortening. However, our studies of the canine bronchial smooth muscle have shown that, as in the hog carotid , normally cycling and latch bridges are present (Jiang et al. 1990). The former, which are active for the first 2s of contraction, cycle at a relatively fast rate and are responsible for effecting 75% of the total isotonic shortening of the muscle. As this occurs within 2s, it is obvious that velocity of shortening could be a limiting factor to shortening; hence velocity becomes an important consideration. The mechanism underlying the increased shortening velocity could be the increased ATP utilization rate in cross-bridge cycling, which can be explained by increased myosin light chain 20 phosphorylation and increased myosin heavy chain ATPase activity itself.

We found no difference in ΔLmax between the groups with and without ATP-γ-s treatment, but an increasesd ΔLmax in the skinned preparations when compared to the intact muscle. This can be explained by the changes in Vo we have discussed above.

Conclusion

We have reported results from a series of experiments which showed that in the skinned tracheal smooth muscle from a ragweed pollen-sensitized canine model, increased maximum shortening velocity and maximum shortening capacity were found. Comparing the result of the intact muscles,

we concluded that both changes in the contractile apparatus and the muscle cell membrane contributed to the increased mechanical properties which led to allergic bronchoconstriction.

In addition, ATP-γ-s was used to study the further relationship between Po, Vo and MLC_{20} phosphorylation. As there were no changes between groups with and without ATP-γ-s treatment, we conclude that the relationship between Po, Vo and MLC_{20} phosphorylation was not a simple linear one, and that beyond a certain level of MLC_{20} phosphorylation, Po and Vo are not affected any more. The mechanism underlying this result needs to be further studied.

Chapter II:ATP utilization in cross-bridge cycling of skinned airway smooth muscle from ragweed pollen-sensitized dogs and littermate controls

Abstract

We have reported that unloaded shortening velocity (Vo) and the phosphorylation of the 20KDa Dalton regulatory myosin light chain (MLC_{20}) of airway smooth muscle were enhanced in our ragweed pollen sensitized canine model of asthma. We also reported that content and total activity of myosin light chain kinase (MLCK) increased but its specific activity remained unchanged in the sensitized group. In the present study we attempted to determine whether the increase of shortening velocity in the treated group was due to the elevation of MLC_{20} phosphorylation and/or changes in enzymatic properties of myosin heavy chain ATPase. ATP-γ-s is believed to be a substrate for myosin light chain kinase but not for actin-activated myosin ATPase and can be used to thiophosphorylate the myosin light chain of chemically skinned smooth muscle. Using the fluorescence method to measure ATPase activity of ATP-γ-s treated smooth muscle, the actin-activated myosin ATPase activity (the rate of ATP utilization in cross-bridge cycling) can be compared between the treatment and control groups. Our results show that at the same level of MLC_{20} thiophosphorylation, which is nearly 100%, the rate of ATP utilization in cross-bridge cycling alone of sensitized canine airway smooth muscle is significantly greater than that of the control ($p<0.05$). We conclude that in physiological circumstances the increased total ATP utilization rate which is due to the increased myosin heavy chain ATPase activity, as well as the increased MLC_{20} phosphorylation permits the increased shortening velocity which may account for allergic bronchospasm.

Literature Review and Background

We have reported that both in tracheal and bronchial smooth muscle the unloaded shortening velocity (V_o) was increased in ragweed pollen-sensitized canine model when compared to its littermate controls. (Antonissen et al. 1979, Stephens et al. 1991, Jiang et al. 1992). In Part I we have just described that the Vo of skinned sensitized tracheal smooth muscle was enhanced as well. We have concluded the reasons for the increased Vo could be, in terms of bioenergetics, due to changes in the contractile apparatus and smooth muscle cell membrane. Waldron et al. have studied the changes in the cell membrane and hence in this study we will focus on the changes in the contractile apparatus. Many studies have reported that cross-bridge cycling rates, as measured by isotonic shortening velocity at small or zero external load, were consistently linearly proportional to cross-bridge phosphorylation in a steady-state contraction in the swine carotid (Aksoy et al. 1982, 1983, Dillon et al. 1982, Singer et al. 1986). This is true in other smooth muscle types (Gerthoffer et al. 1983, Kamm et al. 1985, Weisbrodt et al. 1985), with different types of activation (K^+ depolarization and receptor activation) (Gerthoffer et al. 1983, Rembold et al. 1986, 1988) and at different temperatures (22° and 37° C) (Morland et al. 1986). Many studies have reported that in skinned smooth muscle fibres the level of free Ca^{++} regulated Vo (Arner 1983, Barsotti et al. 1987, Paul et al. 1983). Vo was also found to be dependent on calmodulin concentration (Paul et al. 1983). These findings suggested that Vo was related to the level of myosin light chain phosphorylation, mediated by the activity of Ca^{++}-calmodulin dependent myosin light chain kinase. Arner 1996 also demonstrated that the level of myosin light chain phosphorylation was the major factor determining Vo in the skinned preparations. In our previous work we have demonstrated that 20KDa regulatory myosin light chain (MLC_{20}) phosphorylation of airway smooth muscle (Jiang et al. 1995) was

enhanced in our ragweed pollen-sensitized canine model of asthma. This increase in phosphorylation of MLC_{20} was explained by another study that we conducted which showed that the content of myosin light chain kinase (MLCK) increased but the specific activity of MLCK remained unchanged in sensitized airway smooth muscle (Jiang et al. 1992). It is this which accounted for the increased Vo. However, whether the increase of shortening velocity in the treated group is entirely due to the elevation of MLC_{20} phosphorylation or to changes in the intrinsic enzymatic properties of myosin heavy chain ATPase is not clear. Given full phosphorylation of MLC_{20} and acknowledging that this not only initiates the myosin ATPase activity much like a switch, but also stimulates the enzyme (unlike skeletal muscle) (Stephens et al. 1969), it is possible that the myosin ATPase itself possesses increased intrinsic activity beyond that of simple stimulation. One explanation is that the myosin ATPase activity is the result of the activities of a spectrum of myosin heavy chain isozymes (Kelley et al. 1994). Kelley found that there existed both amino-terminal and carboxyl-terminal alternatively spliced isoforms of the smooth muscle myosin heavy chain (MHC). mRNA splicing at the 3' end generates two MHCs, a 204-KDa isoform (MHC_{204}) and 200-kDa isoform (MHC_{200}). The amino-terminal spliced MHC_{204} and MHC_{200} isoforms are the result of the specific insertion or deletion of seven amino acids near the ATP-binding region in the myosin head at the junction between the 25K and 50K domains. They referred to these isoforms as inserted (MHC_{204}-I; MHC_{200}-I) or noninserted (MHC_{204} ; MHC_{200}), respectively. The inserted isoforms are collectively termed sm-B. Redistribution of these isozymes as a result of sensitization could increase the myosin heavy chain ATPase activity itself (Paul et al. 1991). This is particularly true for sm-B, which is expressed in intestinal, phasic-type smooth muscle, has a higher actin-activated Mg^{2+}ATPase activity and translates actin filaments at a greater velocity in a so-called *in vitro* motility

assay than the noninserted MHC isoform, which is expressed in tonic-type vascular smooth muscle (Kelley et al. 1993, 1994). An increase in the content of sm-B could then account for the increased myosin heavy chain ATPase activity. Other possibilities for the increased myosin heavy chain ATPase activity could be due to the changes in the contents of thin filament related regulatory proteins-calponin and caldesmon. Caldesmon has been proposed to be involved in the regulation of smooth muscle contraction (Furst et al. 1986, Sobue et al. 1982). *In vitro*, caldesmon inhibits the superprecipitation of actomyosin (Sobue et al. 1982) and the actin-activated Mg^{2+}ATPase activity of smooth and skeletal myosin (Chalovich et al. 1987, Dabrowska et al. 1985, Ngai et al. 1984, Smith et al. 1985, Sobue et al. 1985). It also alters the movement of the actin filament on myosin in the *in vitro* motility assay (Haeberle et al. 1992, Okagaki et al. 1991, Shirinsky et al. 1992). Calponin causes an inhibition of actomyosin ATPase as well as an arrest of actin filament movement in in vitro motility assays (Shirinsky et al. 1992, Haeberle 1994, Anderson et al. Unpublished results), in a dose-dependent fashion. Tropomyosin is another regulatory protein that can modulate actin-activated myosin Mg^{2+}-ATPase activity. In addition, recent biochemical data have revealed a close structural correlation between the essential 17KDa myosin light chain (LC_{17}) and the ATPase site on the myosin head (Okamoto et al. 1986). Two isoforms - the basic (LC_{17b}) and acidic (LC_{17a}) light chains have been found in smooth muscle (Cavaille et al. 1986, Hasegawa et al. 1988, Helper at al. 1988). An increased relative content of the basic LC_{17} isoform, is associated with a lower actomyosin ATPase activity (Helper et al. 1988) and Vo (Malmqvist et al. 1991). This could be another reason for the increased myosin heavy chain ATPase activity.

In the present study we will focus on the energetics of smooth muscle contraction to answer the question of whether the increase of shortening velocity in the treated group is due to the elevation

of MLC_{20} phosphorylation and/or changes in enzymatic properties of myosin heavy chain ATPase. ATP utilization in stimulated smooth muscle, at lengths chosen to optimize active isometric force, (Lo), is characterized by a biphasic pattern. A rapid increase in the rate of ATP utilization (J_{ATP}) to about three times the unstimulated rate is followed by decrease to steady state rates of approximately twice the unstimulated rate. The time course and magnitude of changes are dependent on the tissue and stimulus studied, but this pattern has been observed in a variety of preparations (Hellstrand et al. 1982, Paul 1980, Paul 1987, Peterson et al. 1988). J_{ATP} is composed of a component reflecting activation processes (Ca^{++} mobilization and signaling cascades, including cross-bridge phosphorylation) and a component attributable to cross-bridge cycling (actin-activated myosin ATPase activity) (Paul 1989, Wingard et al. 1997). ATP-γ-s is known to be a substrate for myosin light chain kinase but not for actin-activated myosin Mg^{2+}ATPase (Wysolmerski et al. 1991) and can be used to thio-phosphorylate the MLC_{20} (Hoar et al. 1979) of chemically skinned smooth muscle. When the smooth muscle is treated with ATP-γ-s , the actin-activated ATPase activity (the rate of ATP utilization by cycling cross-bridge alone which is subserved by myosin heavy chain ATPase activity) can be separated from that of MLC_{20} phosphorylation. In this study we will employ the NADH fluorescence method (Güth et al. 1986, Griffiths et al. 1980) to measure ATPase activity of ATP-γ-s treated smooth muscle, thus enabling actin-activated myosin ATPase activity to be compared between the treatment and control groups. We should then be able to answer the question of whether the increased Vo seen in sensitized airway smooth muscle is due to elevation of MLC_{20} phosphorylation or changes in enzymatic properties of myosin heavy chain ATPase. Once increased utilization rate by sensitized cross-bridge cycling is established, the reasons can be explored.

Methodology

The NADH fluorescence method was used to determine ATP consumption rate and calculate ATPase activity of an activated smooth muscle combined with the use of ATP-γ-s, and to interpret the results in term of cross-bridge cycling energetics.

In Vivo Sensitization, Smooth Muscle Preparation and Skinning Procedure were performed as previously discribed (Ma, Chapter I).

ATPase Assay

Skinned muscle strips to be used for measurement of ATPase activity were mounted in a muscle lever system designed by Güth (Güth et al. 1986). The strips were mounted between two stainless steel tweezers, one of which was attached to a force transducer and the other to a servo-motor, in a small ($1x1mm^2$) square quartz cuvette containing high-EGTA relaxing solution for 15 minutes and changing to low-EGTA relaxing solution.

The strips were stretched to a length that produced 40-60 mg resting force. This level of passive force was about 10% of maximal active force produced by this smooth muscle preparation. If the passive force of intact canine tracheal smooth muscle is 5-10% of active force, the strips are near the length at which maximal active force is developed (Herlihy et al. 1973, Morland et al. 1988). The mounted strips were perfused with a fresh low-EGTA relaxing solution every 20 seconds until a stable resting force was obtained. Contractions were initiated by introducing the contracting solution, which has the same composition as low-EGTA solution, except for the inclusion of 25μM free Ca^{2+}. In one group of muscle strips the above experiments were conducted after exposure to 1mM ATP-γ-s for 30 minutes in the presence of 25uM Ca^{2+}.

Total ion concentrations were calculated from the desired free ion concentrations by a computer

program based on the multiple ionic equilibrium equations using binding constants. The final solution was checked using a Ca^{2+} -specific ion sensitive electrode (Orion Research Inc., Cambridge, MA, model 93-20).

The hydrolysis of ATP was measured by the NADH fluorescence method, in which ATP is regenerated from ADP and phosphenol pyruvate (PEP) by the enzyme pyruvate kinase (PK) (Güth et al. 1986, Griffiths et al. 1980, Güth et al. 1982). This reaction is coupled to the oxidation of NADH (fluorescent) to NAD (non-fluorescent) and the reduction of pyruvate to lactate by lactate dehydrogenase (LDH) (Takashi et al. 1979).

ATP → ADP + Pi

ADP + PEP → ATP + pyruvate (catalyzed by PK)

pyruvate + NADH → NAD^+ + lactate (catalyzed by LDH)

The solution was excited at 340 nm and the resultant fluorescence was detected at 470 nm by a photometer. The solution in the cuvette was completely changed every 20 seconds. The signal outputs from the lever were digitized by an IBM 486 personal computer, which analyzed and plotted the data using a customized computer program. A linear regression of the records of change in fluorescence collected between 5 and 15s following perfusion was used to calculate ATPase activity (see Figure 4). The signal outputs from the lever were digitized by an IBM personal computer, which analyzed and plotted the data using a customized computer program.

Time Course of MLC_{20} Phosphorylation

The dissected muscle strips were separated into nine groups and the two ends of each muscle strip were fixed for 15 minutes by pins for equilibration in nine dissecting dishes which contained relaxing

solution. Then the solution was quickly changed to contracting solution which has 25μM free calcium and 1mM ATP-γ-s and the muscle strips contracted isometrically. In each of the groups the reaction was stopped after 0, 0.5, 1, 2, 3, 5, 10, 20, 30 minutes respectively by removing the contracting solution and abruptly freezing in an acetone/TCA (5% wt/vol) mixture cooled to -80°C in liquid nitrogen.

The frozen tissue strips were then homogenized in sample buffer which contains 6.4M urea, 10mM DTT, 10mM EGTA, 1mM EDTA, 50mM NaF, 1mM phenylmethylsulfonyl fluoride (PMSF) and urea gel buffer stock. Sample tubes were subjected to centrifugation at 15,000g for 30 minutes.

The nondenaturing 10% polyacrylamide urea gel method, modified from that developed by Hathaway and Haeberle (Hathaway et al. 1985) was employed to separate the nonphosphorylated from the mono-phosphorylated MLC_{20}, as the two possessed different electrical charges. The running tank buffer contained 20mM Tris, 22 mM glycine, 1mM sodium thioglycate, and 1 mM 1, 4 dithiothreitol. All gels were subjected to pre-electrophoresis for 1.5 h at 400 V. A crude extract of 50μl sample was applied to each well. Electrophoresis was conducted at 350V for 18 to 19h at 15°C and was stopped 1h after the bromophenol blue tracking dye exited the gel. The gels were then stained with the help of a silver staining kit (ICN Biomedicals). The band identity was confirmed by Western blotting using anti-MLC_{20} monoclonal antibody (Sigma Immunochemicals).

Proteins were transferred to nitrocellulose sheets under buffer solution containing 25 mM Na_2HPO_4. The blotting was conducted at 1.5amps, 15°C for 1.5h. Nitrocellulose sheets were shaken in blocking buffer consisting of 10 mM Tris saline, 5% bovine serum albumin, and 0.1% gelatin for at least 2 h and then were rinsed 3 times (3 min each) with Tris saline buffer + 0.1% Tween 20 (TBS-T) solution. The sheets were incubated in a monoclonal mouse IgM-anti-smooth muscle MLC_{20} antibody solution

(1:1000; Sigma Immunochemicals) for at least 1h and then washed 3 times with TBS-T. An Amersham blotting detecting kit (RPN 22) for mouse antibodies (biotinylated, species-specific) was used for staining the blots. This method yields highly specific binding of MLC_{20} (see Figure 6).

The developed nitrocellulose sheets were rendered transparent with a plastic embedding method described below (Pharmacia LKB Biotechnology). Polyvinyl chloride write-on films for the overhead transparency were used to sandwich the nitrocellulose sheets. Benzoin methyl ether (0.5g) dissolved in 25 ml trimethylol propane trimethacrylate (2-ethyl-2-(hydroxymethyl)-1, 3-pronpanediol trimethacrylate) was applied to one half of the film. The nitrocellulose sheet and the other half of the film were then placed on top of it. The embedded sheet was pressed with a roller to squeeze out the air bubbles between the plastic films. The sandwich was then irradiated with long wave UV light for 3 min for each side to polymerize the sheet. The embedded transparent sheets containing band images were then scanned with an LKB laser densitometer and the images of MLC were analyzed using methods similar to those described before (Jiang et al. 1991). The ratio of phosphorylated to total MLC_{20} was obtained.

MLC_{20} Quantification

The MLC_{20} content in tissue homogenates of sensitized and control tracheal smooth muscle was obtained by quantitative 15% mini SDS-PAGE. The protein loads for both control and sensitized groups were the same ($5\mu g$). Bands of MLC_{20} were identified and quantified by laser scanning and comparison with a SDS-page molecular weight standards-low (Bio-Rad Laboratories) on the same gel (Figure 5). The identity of the bands was also confirmed by Western blotting employing a 1:1000 monoclonal anti-smooth muscle MLC_{20} antibody (Sigma Immunochemicals). Images of scanned bands were restored in a computer, which enabled us to obtain the volumes of the bands.

Statistical Analysis

All values were given as means ± SE with the number of observations within parentheses. The mean values of MLC_{20} content and MLC_{20} phosphorylation level and the standard error were calculated by the SigmaPlot program. The mean values from sensitized and control groups were compared using unpaired two-tailed t test with p set at 0.05. The time course of ATPase activity from sensitized and control muscles; with and without ATP-γ-s (four groups) were analysized by the NCSS6.0 MANOVA Report which enables a one way analysis of variance. This was combined with a Duncan's new multiple range test to compare means.

Results

MLC_{20} Content of Sensitized and Control Tracheal Smooth Muscle (STSM and CTSM)

Through 15% mini SDS-PAGE, we obtained the scanned volume of the MLC_{20} content (see Figure 5). In sensitized TSM it was 1.7414 ± 0.1497, while in control group it was 2.0769 ± 0.4466. Statistical analysis using SigmaPlot software showed that there was no significant difference ($p>0.05$) in MLC_{20} content between CTSM and STSM. If the MLC_{20} thiophosphorylation levels are also same in both groups, any difference in ATP utilization rate in cross-bridge cycling between the two groups, will not be due to difference in MLC_{20} phosphorylation level but to changes in myosin heavy chain ATPase itself.

Time Course of MLC_{20} Phosphorylation in ATP-γ-s Treated Skinned CTSM and STSM

Figure 6 shows MLC_{20} phosphorylation in ATP-γ-s treated skinned CTSM and STSM. The upper row of bands was non-phosphorylated 20KDa myosin light chain. The middle row of bands was mono-phosphorylated 20KDa myosin light chain. The lower one was the di-phosphorylated 20KDa myosin light chain. In the present study we used the proportion of phosphorylated 20KDa myosin

light chain content to total myosin light chain content as the MLC_{20} phosphorylation level. The calculated data are depicted in Figure 7 as the time course of MLC_{20} phosphorylation in ATP-γ-s treated skinned CTSM and STSM. We found that MLC_{20} phosphorylation of the sensitized group had a faster increase trend with time than the control group. In both of the two groups, 5 minutes after stimulation with 25 μM Ca^{++}, myosin light chains were thiophosphorylated and reached the same maximum level (100%). Since the thiophosphorylation of myosin light chain 20 is not reversible, the ATP utilization we measure after ATP-γ-s treatment should be the ATP utilized in cross-bridge cycling only and reflects the myosin heavy chain ATPase activity itself.

In this study the time course of MLC_{20} phosphorylation in the absence of ATP-γ-s treated skinned CTSM was also measured. Figure 8 shows the experimental record and Figure 9 plotted the scan values of Figure 8 and it shows that without ATP-γ-s the maximal phosphorylation level of CTSM also reaches a high level of ~83% which however is less than the 100% seen in the with ATP-γ-s treated skinned muscle.

Time Course of ATPase Activity

In this study the ATPase activity before Ca^{++} stimulation was termed basal ATPase activity. We normalized measured ATPase activity with respect to basal ATPase activity. The ratio of stimulated ATPase activity to basal ATPase activity was used to indicate the relative value of ATPase activity since the absolute value was hard to determine. Figure 10 shows the time course of ATPase activity from ATP-γ-s treated CTSM and STSM. Since in this experiment ATP-γ-s was used, the ATPase activity we measured was the actin-activated myosin Mg^{2+}-ATPase activity (solely originating from cross-bridge cycling). We found that the actin-activated ATPase activity of sensitized TSM was significantly higher than that of the controls ($\alpha<0.05$). The mean values of both curves were

calculated (see Table 3). They were 3.0934±0.3942 (arbitrary units) in the sensitized group and 2.3140±0.3110 (arbitrary units) in the control group. Thus the actin-activated Mg^{2+}-ATPase activity of STSM increased by 33.68%. In this study we also measured the time course of ATPase activity in CTSM and STSM which had not been treated with ATP-γ-s (see Figure 11). Since ATP-γ-s was not used here, the ATPase activity we measured was the total ATP utilization rate. We found it was also higher in the sensitized group ($\alpha<0.05$). The mean values were 3.4860±0.3228 (arbitrary units) in STSM and 2.5631±0.1563 (arbitrary units) in CTSM. The total ATP utilization rate of STSM increased by 36.01% (see Table 3). When we compared the total ATPase activity between treated or not treated with ATP-γ-s groups (see Figure 12), we found that both in CTSM and STSM the ATPase activity of groups untreated with ATP-γ-s (the total ATPase activity) was significantly higher than that of treated groups (actin-activated ATPase activity). However the ATP-γ-s treated group reached steady-state faster than the untreated group. The difference of ATPase activity between the two groups could be due to the ATP utilized in the myosin light chain kinase and myosin light chain phosphatase cycle (pseudo-ATPase activity). The mean values were also calculated (see Table 3). They were 0.3926±0.0828 in STSM and 0.2491±0.0615 in CTSM, an increase of 57.61% in STSM. In Figure 13 we plotted the previous four curves together and comfirmed the above results. In this study Duncan's new multiple-comparision test was also used to analyze the four curves and showed that all the point values on each curve were same. This demonstrated there was no decrease of ATPase activity during the time course of muscle contraction which indicated that latch-bridges were not active in skinned preparations.

Discussion

In 1975 it was shown that the phosphorylation of chicken gizzard myosin was associated with an

increase in its actin-activated Mg^{2+}-ATPase activity (Bremel et al. 1974, Sobieszek 1977), suggesting that the actin-myosin interaction might be regulated via phosphorylation of the myosin molecule. These observations were confirmed and extended to show that phosphorylation of myosin occurred in a variety of smooth muscles (Hartshorne et al. 1981). A linear relationship between the extent of myosin phosphorylation and the actin-activated Mg^{2+}-ATPase activity of gizzard actomyosin and myofibrils was observed (Sobieszek et al. 1977). The increased myosin light chain 20 phosphorylation in ragweed pollen-sensitized canine airway smooth muscle that we have demonstrated could account for the increased maximum shortening velocity in this muscle. However, the myosin heavy chain ATPase itself may play an intrinsic role in the increased Vo as well. The energetics of muscle contraction were studied here to test whether this hypothesis was true.

In the present study we determined that the contents of myosin light chain 20 were the same in sensitized and control tracheal smooth muscle. It was also found that MLC_{20} was 100% thiphosphorylated about 5 minutes after stimulation with Ca^{++} in both groups treated with ATP-γ-s. Therefore we concluded the ATP utilization by the muscle in myosin light chain thiophosphorylation should be the same in both groups. On this basis we measured the ATP utilization rate commencing at 10 minutes after stimulation in both groups. It represented the ATP utilization by the cycling cross-bridges alone. The difference in ATP utilization rate between sensitized and control groups must be due to the activity of the myosin heavy chain ATPase itself. It was also found that the sensitized group had a faster increase trend of MLC_{20} phosphorylation than the control group. This could be explained by the increased myosin light chain kinase content in the sensitized group we had demonstrated before. However, given enough time, both muscles reached the same maximum phosphorylation level (100%). The time course of myosin light chain 20 phosphorylation in the

absence of ATP-γ-s was also measured in the control group. We obtained a maximum level of myosin phosphorylation of about 83.61% which explained the unchanged Po and Vo between the with and without ATP-γ-s treated groups in the previous mechanical studies.

Our assay of ATPase activity monitors the loss of NADH fluorescence that is coupled to ATP utilization via an enzyme system. This method measures the total ATPase activity in the detergent-skinned tracheal smooth muscle rather than a specific ATPase. In these experiments, mitochondrial ATPases were inhibited by the inclusion of sodium azide (NaN_3), and adenylate deaminase was inhibited by the inclusion of P^1, P^5-di(adenosine-5') pentaphosphate in all solutions. Total tissue ATPase, however, may be composed of not only actin-activated myosin ATPase but, in addition, of sarcolemmal and sarcoplasmic reticular Ca^{2+}-ATPases, Na^+-K^+-ATPase, or pseudo-ATPases composed of MLC kinase and phosphatase activities. Moreland's lab (Zhang Yawen et al. 1994) have proved that Ca^{2+}-stimulated ATPase activity was not affected by the sarcolemmal Ca^{2+}-ATPase inhibitors quercetin (Stephenson et al. 1985) or cisapride (Den Hertog et al. 1986), by ouabain, an inhibitor of the Na^+-K^+-ATPase and by thapsigargin (Thastrup et al. 1989), an inhibitor of sarcoplasmic reticulum (SR) CaATPase. Therefore the ATPase activity we measured in these experiments should be only composed of the actin-activated myosin ATPase activity and the pseudo-ATPase activity. In this study, we measured the ATPase activity of four groups- sensitized and control each with and without ATP-γ-s treatment. Since ATP-γ-s can thiophosphorylate MLC_{20} irreversibly, the ATPase activity we measured in the ATP-γ-s treated group should be only the actin-activated ATPase activity while in the ATP-γ-s untreated group it should be the total ATPase activity which includes not only the actin-activated myosin ATPase activity but also the pseudo ATPase activity.

In this study we demonstrated that when ATP-γ-s was used, the ATPase activity of muscles obtained from sensitized dogs was significantly higher than that of the controls. Since the MLC_{20} contents and the MLC_{20} thiophosphorylation levels were the same between the two groups, the same amount of thiophosphorylated myosins thus operated in the cross-bridge cycling and the difference of ATPase activity between two groups could not be due to the difference in MLC_{20} phosphorylation but only to changes in the actin-activated myosin heavy chain Mg^{2+}-ATPase activity. From these results we were able to conclude that the myosin heavy chain ATPase activity itself was increased in sensitized tracheal smooth muscle. However the reason for the enhanced myosin heavy chain ATPase activity is not clear. It is known that the redistribution of myosin heavy chain isozymes could lead to changes in myosin heavy chain ATPase activity (Paul et al. 1991). In relation to this we have demonstrated that the distribution of myosin heavy chain isozymes in the two groups of airway smooth muscles was unchanged (Stephens et al. 1988) and therefore this could not be the reason for the enhanced myosin heavy chain ATPase activity. The other reasons could be the changes in the contents of calponin (Shirinsky et al. 1992, Haeberle 1994, Anderson et al. Unpublished results) and caldesmon (Chalovich et al. 1987, Dabrowska et al. 1985, Ngai et al. 1984, Smith et al. 1985, Sobue et al. 1985) since they were reported to have inhibitory effects on the myosin heavy chain ATPase activity. In addition to that, higher level of sm-B, a myosin heavy chain isozyme was found to be associated with higher myosin heavy chain ATPase activity (Kelley et al. 1993, 1994) and increased basic myosin light chain 17 was reported to be associated with lower actomyosin ATPase activity (Helper et al. 1988; Malmqvist et al. 1991). Therefore the contents of calponin, caldesmon, tropomyosin, sm-B and MLC_{17b} will be measured in the near future in both sensitized and control groups to investigate further the causes for the enhanced myosin heavy chain ATPase activity. In

physiological circumstances MLC_{20} was not thiophosphorylated and therefore we concluded that both increased MLC_{20} phosphorylation and myosin heavy chain ATPase activity contributed to the increased shortening velocity which we found in ragweed pollen-sensitized canine tracheal smooth muscle. To put it another way, both the endogenous ATPase activity and the extent of its stimulation by phosphorylation were increased.

In the ATP-γ-s untreated group the ATPase activity of the sensitized tracheal smooth muscle was also higher than that of the controls. Since ATP-γ-s was not used, this ATPase activity was the total ATPase activity which included the ATP utilization in cross-bridge cycling and in MLC_{20} phosphorylation/dephosphorylation. The above result allowed us to calculate that ~89% of the total ATP was used in cross-bridge cycling and 11% was used in MLC_{20} phosphorylation /dephosphorylation. This finding was similar to that presented by Moreland (Zhang et al. 1994) using permeabilized swine carotid arteries and Hellstrand and Arner (Hellstrand et al. 1985) using permeabilized guinea pig taenia coli which demonstrated that a preponderant amount of total ATP utilization was due to cross-bridge cycling, and only a small part was due to MLC kinase and phosphatase activity. However this finding was inconsistent with the model for smooth muscle regulation proposed by Hai and Murphy (Hai et al. 1988), which requires a significant proportion of total cellular ATPase activity to be due to MLC kinase-MLC phosphatase activity.

Both in sensitized and control groups we found that the total ATPase activity was higher than the actin-activated ATPase activity alone. The difference between them was due to the pseudo ATPase activity. When the pseudo ATPase activity was compared between the sensitized and control groups, we also found it was higher in the sensitized group (see Figure 12 and Table 3) which further added validity to our previous result that MLC_{20} phosphorylation was increased in our sensitized model.

In this study Duncan's new multiple-comparision test was also used to analyze the four curves of time course of ATPase activity and showed that all the point values on each curve were same. This result demonstrated there was no decrease of ATPase activity during the time course of muscle contraction which indicated latch bridges were not operating during contraction in the skinned preparations. This result was not consistent with the latch-bridge model (Hai and Murphy 1988) however it was consistent with the result of Kerrick's lab (Kenney et al. 1990) which found that high levels of tension generated at low levels of light-chain phosphorylation are associated with high levels of ATPase activity from skinned chicken gizzard smooth muscle.

Conclusion and Future Work

The present study demonstrated that myosin heavy chain ATPase activity as well as MLC_{20} phosphorylation were enhanced in our ragweed pollen-sensitized tracheal smooth muscle which provided insight into the mechanism of increased shortening velocity in such model. Future work will be in two directions: one will be the comparision of the message levels of MLCK content between sensitized and control groups so as to account for the increased protein content of MLCK. The other direction will be an investigation of the reasons for the increased myosin heavy chain ATPase activity which includes the measurement of contents of certain proteins that influence the myosin heavy chain ATPase activity. Both of studies will contribute to elucidation of the mechanism of allergic bronchospasm.

Chapter III: Length dependent ATP consumption rate in smooth muscle: evidence for plasticity

Abstract

In the field of smooth muscle contractility it has been shown that the mechanism underlying smooth muscle's ability to function over a large length range could be radically regulated by plastic restructuring of the contractile filaments inside the cells. According to the plastic theory enunciated by Ford's group (Pratusevich et al. 1995) active tension generated by smooth muscle is independent of length. This behavior is completely different from that in skeletal muscle where the length-tension relation is characteristically described by the Frank-Starling Law and is elicited by subjecting the smooth muscle to the process of adaption. Consequences of this structural alteration may be reflected by the changes in mechanical and energetic properties of the muscle. Increased power output associated with length increase would suggest that additional myosin filaments may be recruited in the contraction. This increased power (active tension x velocity) is the result of a constant active tension at lengths ranging from 0.5Lo to 1.5Lo, but a progressively increasing Vo with increasing length. Measurements of force and ATP hydrolysis indicated that ATP consumption, but not isometric force, increased with increase in muscle length. The results support a model where more myosin thick filaments are laid down in series when a muscle is elongated from 0.5Lo to 1.5Lo.

Literature Review and Background

Large volume changes of hollow viscera such as bowel, bladder, and uterus show that smooth muscle has a large functional length range. It has been shown that in rabbit bladder the smooth muscle cells lining the bladder wall undergo at least a 7-fold length change reversibly under physiological conditions (Uvelius 1976). This ability to function over such a large length range cannot be accommodated by the fixed filament-lattice model of skeletal muscle (Huxley et al. 1954, Huxley et al. 1954, Gordon et al. 1966). A new model for smooth muscle contraction therefore has to be sought. No regular repeating structures similar to the sarcomeres of striated muscle have ever been found in smooth muscle. Even the existence of myosin thick filaments in smooth muscle cells was found to be evanescent (Shoenberg et al.1969, 1976, Godfraind-Debecker et al. 1988, Gillis et al. 1988, Watanabe et al. 1993), although some found that the thick filaments were constantly present (Cooke et al. 1972, Garamvolgyi et al. 1971, 1973, Somlyo et al. 1973, Somlyo et al. 1981). Compared to those in skeletal muscle, thick filaments in smooth muscle appear to be much more labile. This apparent lability has led to a hypothesis (Pratusevich et al. 1995) that the muscle is able to undergo drastic structural changes including rearranging, or even re-synthesizing "contractile units", which in smooth muscle are likely to consist of myosin thick filaments and adjacent actin thin filaments, in response to external demand. This postulates the muscle as being plastic and it adapts to different lengths by changing the number of contractile units in series, possibly by laying down varying numbers of thick filaments in series. This conclusion is supported by experiments showing that smooth muscle shortening velocity, power-output and compliance all increase with increase in muscle length (Pratusevich et al. 1995). If indeed more contractile units are recruited when smooth muscle adapts to longer lengths, an increase in adenosine triphosphate (ATP) consumption by the

muscle is expected. Hence we measured simultaneously the muscle force and ATPase rate at different muscle lengths.

Methodology

Canine trachealis was used in the present experiments because its cells are arranged in long, straight bundles that span the length of the preparation (Stephens et al. 1969) and there is a large length range (at least 3-fold) over which the isometric active force is constant (Pratusevich et al. 1995). Six strips of trachealis from 2 dogs (three from each) were used for the experiments. Each muscle strip was chemically "skinned" to allow large molecules like ATP and NADH to diffuse in. The skinning solution was the same as the relaxing solution which contained 5mM EGTA, 20mM imidazole, 45mM K-acetate, 6.1mM $MgCl_2$, 5.6mM Na_2ATP, 1mM dithiothreitol, 1mM NaN_3 except that 1% (by volume) of Triton X-100 was added. The muscle preparation was treated in the skinning solution for 2h at room temperature to partially remove the cell membrane. Rate of ATP consumption and isometric force produced by the muscle were measured simultaneously in an apparatus designed by Konrad Güth (Güth et al. 1986). One end of the muscle was fixed to a stationary arm, the other end was attached to a force transducer. Slipped over the muscle bundle was a quartz cuvette, through which the muscle was illuminated at 340nm. Hydrolysis of ATP was measured by the NADH (a reduced form of nicotinamide adenine dinucleotide) fluorescence method where ATP is regenerated from ADP and phosphoenol pyruvate by the enzyme pyruvate kinase. This reaction is coupled to the oxidation of NADH to NAD and the reduction of pyruvate to lactate by lactate dehydrogenase (Takashi et al. 1979, Griffiths et al. 1980, Kenney et al. 1990). For each mole of ADP produced by the muscle, an equal amount of the fluorescent NADH is oxidized to the non-fluorescent NAD, and the change in fluorescence intensity is detected at 470nm wavelength.

Figure 14A shows calcium-activated isometric force and rate of ATP consumption by the muscle in the plateau region of a contraction. Activation solution was the same as the relaxing solution except that it contained 0.1mM EGTA and 25μM of free calcium. A rinse solution was used just before the muscle was activated to lower the concentration of EGTA. The composition of the rinse solution was the same as the relaxing solution except the EGTA concentration was 0.1mM. All solutions contained 5mM phosphoenol pyruvate, 0.2mM P^1, P^5-di (adenosine-5') pentaphosphate, 0.6mM NADH, 70U/ml lactate dehydrogenase, 50U/ml pyruvate kinase. The pH of the solutions were adjusted to 7.0 at room temperature. The solution inside the cuvette was changed every 25s to replenish both ATP and NADH, hence the saw-tooth shaped record. In Figure 14B, the saw-tooth record in Figure 14A was expanded for detailed examination. The change of ATP concentration over a 25s period was taken as the intersects of a straight line fitted to the digitized record during the period of ATP depletion, and the two imaginary vertical lines marking the 25s interval. The calculation was repeated for each of the seven "saw-teeth" for each muscle strip, the results were averaged and used as a single observation for statistical purposes. All values were given as means ± SE with the number of observations within parentheses.

Results

Figure 15 shows isometric force and ATPase rate as function of muscle length. In these experiments, measurements were made at two muscle lengths, L_{ref} and 1.5 L_{ref} . L_{ref} is defined as the muscle length at which the resting tension just starts to increase with stretch. The resting tension at L_{ref} was between 0 and 2% of isometric force. A 30-min period was allowed for the muscle to rest in between activations. As shown in Figure 15, active isometric force remained the same at both L_{ref} and 1.5 L_{ref}, whereas the ATPase rate increased by 33% at 1.5 L_{ref} . The absolute values of muscle

forces and ATPase rates at the two lengths and in both active and resting states are listed in Table 5. Six muscle strips were used in the experiments. The sequence of length change in the experiments was chosen so that 3 muscle strips contracted from long to short, and the other 3 from short to long. No difference was found between the two groups. The data from the two groups were averaged.

Discussion

The results indicate that increasing smooth muscle length is associated with an increase in energy consumption, suggesting that as the result of adaption, additional myosin filaments had been recruited to participate in the contractile process. This process was reversible, that is, when muscle was set at a shorter length, the ATPase rate decreased, suggesting that some myosins had been excluded from the contractile process. The previous finding that mechanical power output of smooth muscle varied with muscle length (Pratusevich et al. 1995), therefore, is rooted in the muscle's ability to increase or decrease energy consumption according to cell length.

The power output was found to increase 230% over a 3-fold length change (Pratusevich et al. 1995), which is equivalent to a 38% power increase with the 50% length increase used in the present experiments. The power increase is less than 50% and therefore is not directly proportional to the length increase. There are several reasons for the muscle to deviate from this "ideal" direct proportionality. One is that the method used for attaching the muscle strips to the force transducer and servo motor produced crushed ends with dead cells that did not generate shortening or power. Another reason is that the plastic restructuring of the contractile apparatus inside the cells may not be 100% efficient (Pratusevich et al. 1995). The present finding of a 33% increase in the rate of ATP hydrolysis with a 50% length increase is less but comparable to the previous finding of 38% increase of power output with the same amount of length increase. In the previous experiments, carbon

granules were used to mark the central segment of the muscle strip (Pratusevich et al. 1995), so that the central segment length could be monitored and used in the muscle-length versus power plot. This procedure excludes the stray compliance from the ends of the muscle preparation. In the present experiments, central segment markers were not used because the markers may have interfered with the fluorescence measurement. Inclusion of the stray compliance in the measurements has an effect of reducing the slope of the muscle-length versus Δ[ATP] plot (see Figure 15), because the compliance is highly non-linear, being much less stiff at short length where resting tension is low. This may explain some of the quantitative discrepancy between the previous mechanical measurements (Pratusevich et al. 1995) and the present energetic measurements. Other factors also produced an underestimation of the change in ATPase rate at different muscle lengths. Due to a limitation of the apparatus, fluorescence measurement could only be made over a segment of the muscle strip. The total fluorescence emitted by the muscle was calculated as the fluorescence per unit muscle length times the total length of the muscle. The signal detection window had a dimension of about 2mm in height and 1mm in width, covering a 1mm segment of the horizontally laid muscle strip (about 1/5 to 1/3 of the total length) and a 2mm vertical space inside the cuvette. A relatively large cuvette (2mm x 2mm inside cross-section) was used in the present experiments to minimize measurement artifacts due to muscle volume change associated with length change. When a muscle shortens, its cross-sectional area increases. This has dual effects on the fluorescence measurement. One is that the solution surrounding a muscle segment is reduced because of the finite cross-sectional area of the cuvette, the other is that the background light is reduced, because the "fatter" muscle now blocks more of the excitation illumination. Since the fluorescence signal was taken as the change in light emitted at 470nm divided by the background light, both effects will increase the signal used to

indicate fluorescence change and therefore result in an overestimation of ATPase rate at short muscle length. The muscle strips used in the present experiments had cross-sections of about 0.1-0.15mm^2, less than 4% of the cuvette cross-section. The error due to muscle length change therefore is small and was not corrected.

Although the resting force at short muscle lengths was negligible, the same force at long lengths was not (Table 4). This has raised the question of whether the high resting force is associated with the increased energy consumption. The measured resting ATPase rates suggest otherwise. The resting ATPase rate was low compared to the active rate, in some cases the signal was less than the noise level. From the estimated upper limit of the resting rates, it can be concluded that the high resting force associated with long lengths does not account for the increased ATPase rate seen in long, active muscle. Previous study (Pratusevich et al. 1995) indicated that in the canine trachealis, the passive resting force can be largely eliminated by carefully dissecting away the extracellular connective tissue. This is consistent with the notion that the resting force is borne by non-energy consuming structures.

One likely explanation for the length-dependent rate of ATP hydrolysis in smooth muscle is that there are extra myosins in the cytosol, either in monomeric form or in thick filaments, that are ready to be incorporated serially into the contractile apparatus when the muscle is lengthened. A reverse process would allow the myosins to be extruded from the contractile apparatus when the muscle is adapted at short lengths. Myosins outside of the contractile apparatus should remain inactive. It is not clear how would this dynamic exchange of myosin work within the muscle. The observation that phosphorylation occurs during activation (Sobieszek et al. 1977, Hartshorne et al. 1980, Adelstein et al. 1980), however, suggests that phosphorylation of the light chain may play a role in the

restructuring of the contractile apparatus. The length-independent isometric force suggests that the number of active myosin cross-bridges per cross-section of muscle is not reduced when the muscle is lengthened. The total number of active cross-bridges in a muscle cell therefore must increase when the muscle is adapted to longer lengths. This conclusion is consistent with the energetic measurements. The absence of force change with length change further suggests that restructuring of the contractile apparatus occurs in such a way that more contractile units are appended to the existing units in series, excluding the alternative explanation of contractile units being laid down in parallel.

Conclusion

The present study showed that ATP consumption, but not isometric force, increased with increase in muscle length. Along with the previous results that smooth muscle shortening velocity, power-output and compliance all increase with increase in muscle length, it supported the plasticity theory of smooth muscle which showed that more myosin thick filaments were laid down in series when a muscle was elongated from 0.5Lo to 1.5Lo. It is this which enables smooth muscle to function over such a large length range that cannot be accommodated by the fixed filament-lattice model of skeletal muscle.

Summary

The energetic studies by NADH fluorescence method is a useful way to investigate the mechanisms of smooth muscle contraction. In the present work, we found 1) increased maximum shortening velocity and maximum shortening capacity in the skinned tracheal smooth muscle from a ragweed pollen-sensitized canine model. Comparing the result of the intact muscles, we concluded that both changes in the contractile apparatus and the muscle cell membrane contributed to the increased mechanical properties which led to allergic bronchoconstriction. NADH fluorescence method was then used to demonstrated that increased myosin heavy chain ATPase activity as well as MLC_{20} phosphorylation of sensitized tracheal smooth muscle were the mechanisms. 2) length dependent ATP consumption rate in smooth muscle. It proved the plasticity theory in such muscle where more myosin thick filaments were laid down in series when a muscle was elongated from 0.5Lo to 1.5Lo. It is this plasticity property which enables smooth muscle to function over such a large length range that cannot be accommodated by the fixed filament-lattice model of skeletal muscle.

Contributions of this work: 1) provided insight into the mechanisms of allergic bronchospasm. 2) provided evidence of the plasticity theory in smooth muscle.

Future work will be focused on two directions: one will be the comparison of the message levels of MLCK content between sensitized and control groups so as to account for the increased protein content of MLCK. The other direction will be an investigation of the reasons for the increased myosin heavy chain ATPase activity which includes the measurement of contents of certain proteins that influence the myosin heavy chain ATPase activity. Both of studies will contribute to further elucidation of the mechanism of allergic bronchospasm.

Figure 1. Typical experimental records of Po, Vo, ΔLmax measurements from a single experiment are shown. The upper panel is the time course of force trace. After several minutes of equilibration, the muscle was stimulated by 25μM Ca^{2+} and the force increased until it achieved a steady state. Quick release was then started to suddenly decrease the force to 5% of maximal isometric force and the Vo could be measured from analysis of the slow transient. The middle panel shows the muscle shortening trace. ΔLmax could be directly measured from the change of length trace. The lower panel shows the amplified first derivative trace obtained from the length trace. The lowest point was determined to be the Vo.

Figure 1: Experimental record of Po, Vo, ΔLmax measurement

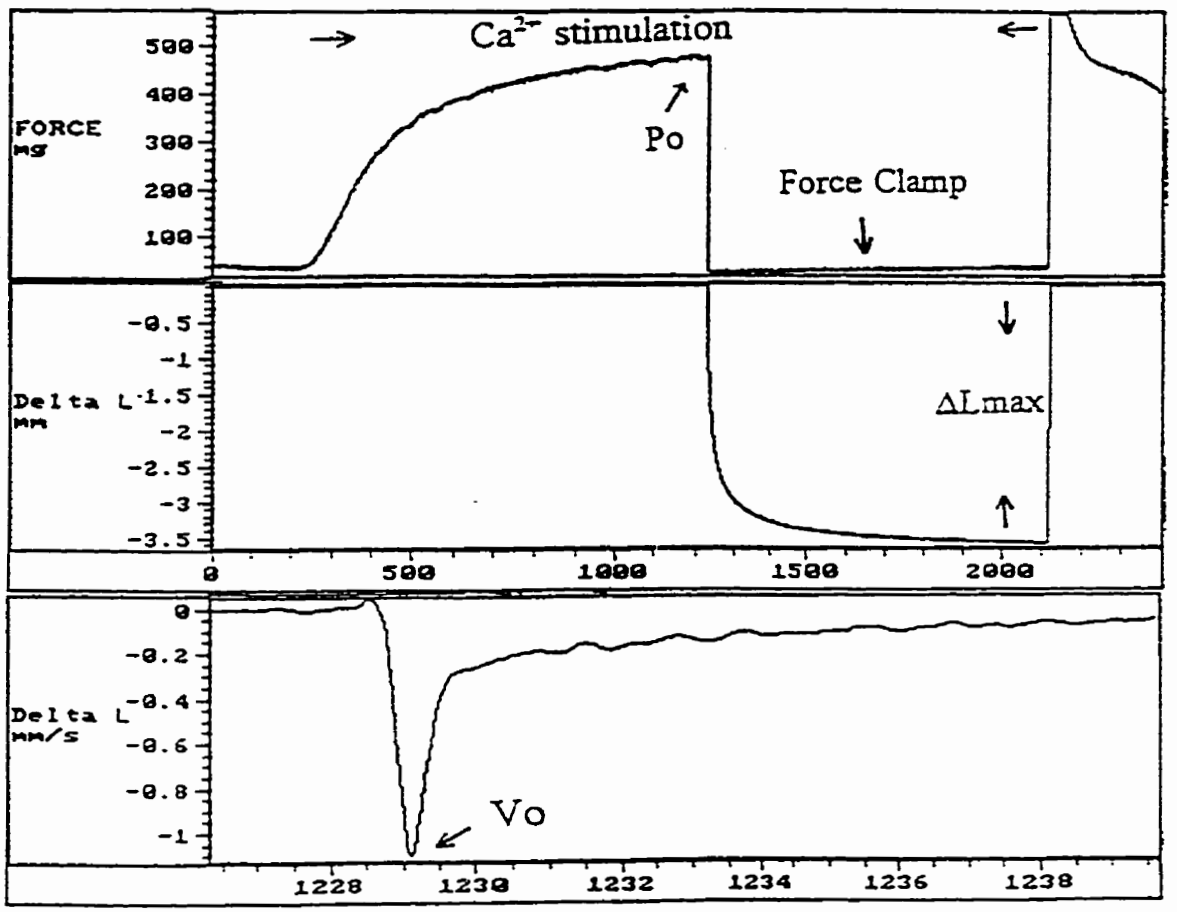

Figure 2. Maximum isometric active force (Po) of skinned control (CTSM) and sensitized (STSM) tracheal smooth muscle from with and without ATP-γ-s treated groups are shown. Means and standard error bars are plotted. w=with; w/o=without. Po was normalized to cross-sectional area. Whether ATP-γ-s was used or not, there was no significant difference of the Po between STSM and CTSM ($p>0.05$) which reflected that Po was an insensitive indicator of bronchoconstriction. Po also showed no difference between with and without ATP-γ-s treated groups ($p>0.05$) which showed a highly non-linear relationship between isometric force and myosin light chain phosphorylation.

Figure 2: Maximum Isometric Force of Skinned CTSM & STSM

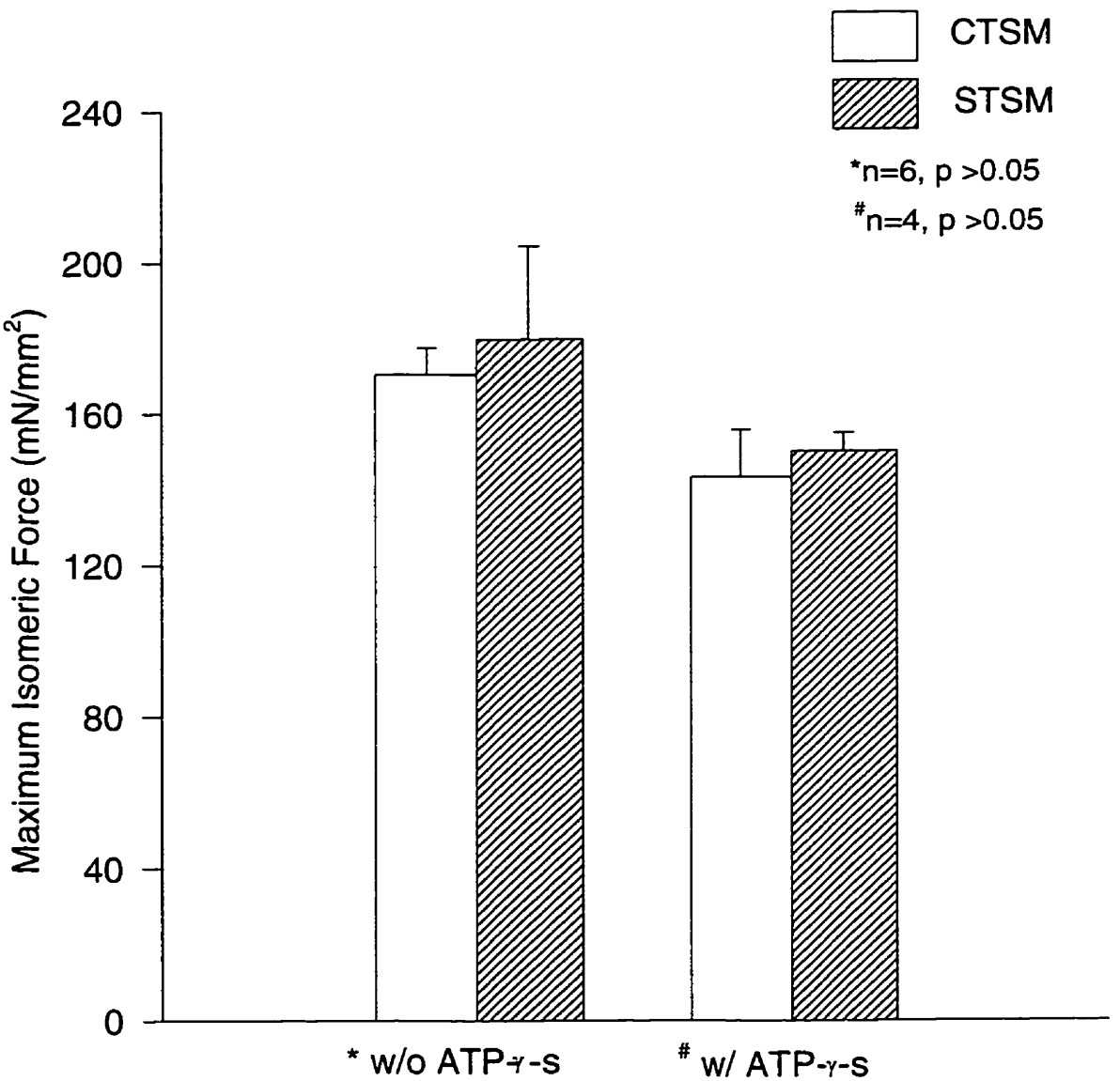

Figure3. Maximum shortening velocity and capacity were compared between sensitized and control tracheal smooth muscle in both with and without ATP-γ-s treated groups. The left panel shows the ΔLmax and the right panel shows the Vo. Open bars represent the control tracheal smooth muscle. Filled in bars represent the sensitized tracheal smooth muscle. Means and standard error bars are plotted. w=with; w/o=without. ΔLmax was normalized to %Lo and Vo was normalized to %Lo/sec. Both ΔLmax and Vo of the sensitized tracheal smooth muscle were significantly higher than those of the controls ($p<0.05$) in both with and without ATP-γ-s treated groups. However, when the ΔLmax and Vo were compared between with and without ATP-γ-s treated groups, they showed no significant difference ($p>0.05$). The increased Vo in STSM could be responsible for the bronchoconstriction.

Figure 3: Increased Maximum Shortening Capacity and Velocity in Skinned STSM

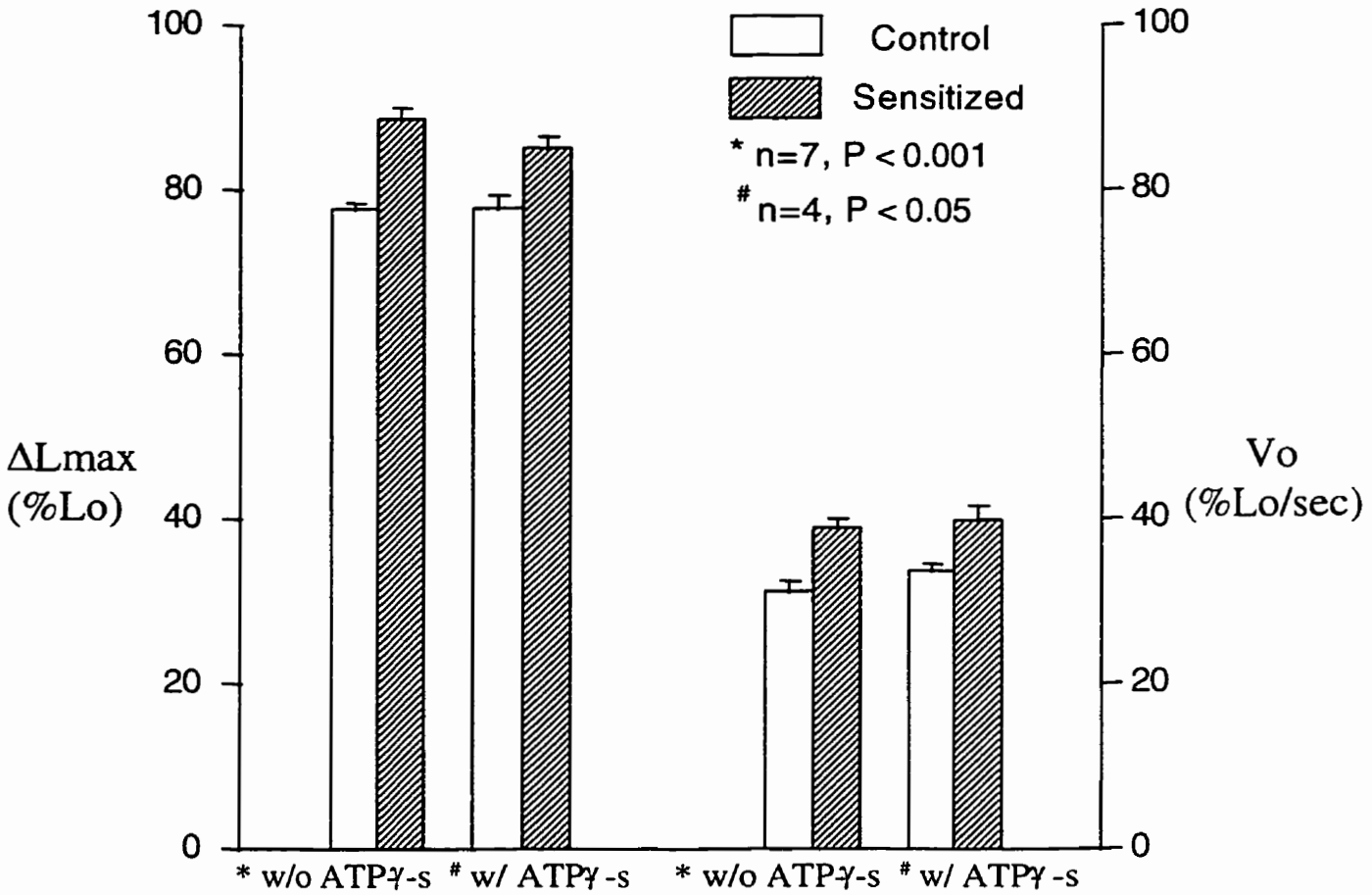

Figure 4. Method for measuring NADH consumption, which is an index of ATP utilization by the cycling cross-bridge, in a "skinned" tracheal smooth muscle strip. NADH is added to the quartz cuvette which holds the skinned (Triton X-100 demembration) smooth muscle. Each oscillation represents consumption of the NADH. The inset in the lower panel is a "blow-up" version of a single oscillation. The slope of the descending limb of this oscillation (d[NADH]/dt) represents the consumption record for the entire contraction and is an index of actin-activated myosin Mg^{2+}-ATPase activity. This record was obtained from a single strip of antigen-sensitized muscle treated with ATP-γ-s which fully phosphorylates the regulatory 20KDa myosin light chain (MLC_{20}).

Figure 4: NADH fluorescence record

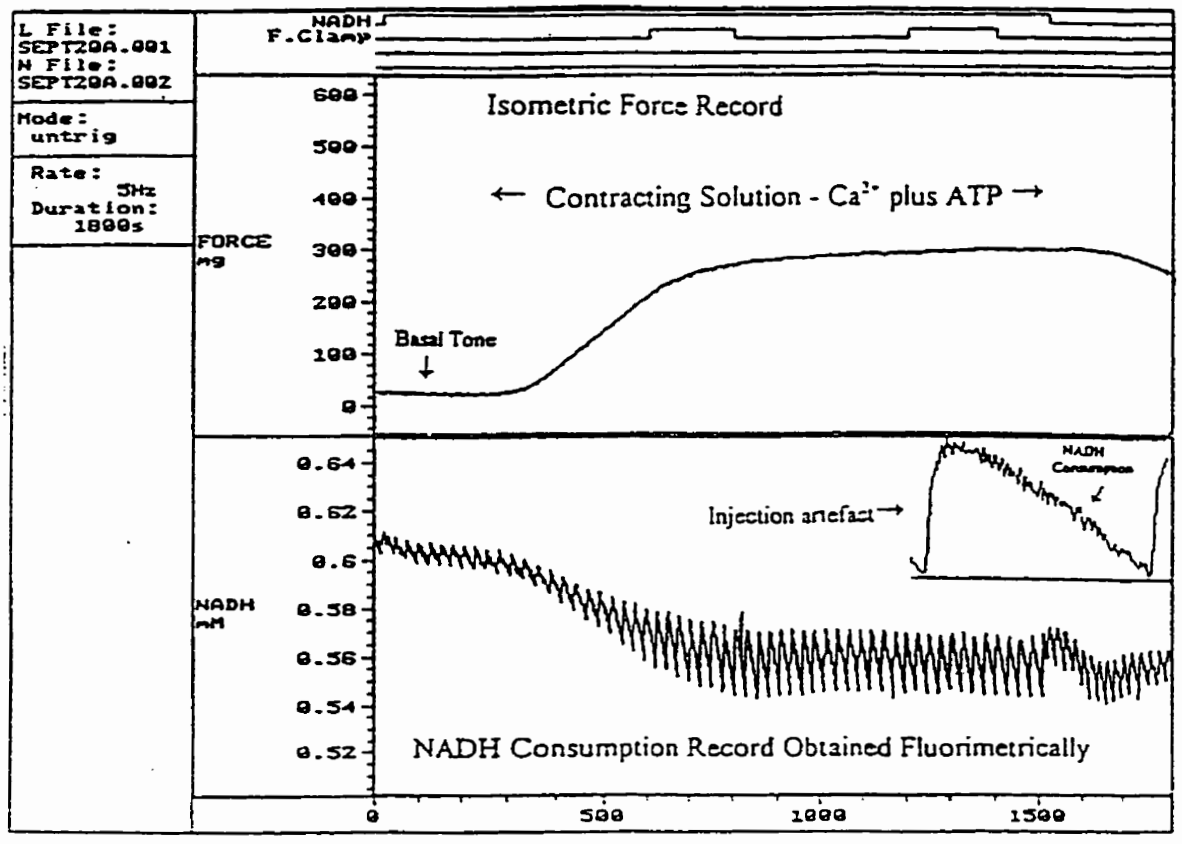

Figure 5. 20KDa myosin light chain contents in CTSM and STSM are shown. C=control; S=sensitized. The MLC_{20} content in tissue homogenates of sensitized and control tracheal smooth muscle was obtained by quantitative 15% mini SDS-PAGE. The protein loads for both control and sensitized groups were the same (5ug). The identity of the bands was confirmed by Western blotting employing a 1:1000 monoclonal anti-smooth muscle MLC_{20} antibody (Sigma Immunochemicals). The volumes of the bands were calculated (CTSM=2.0769$\pm$0.4466; STSM=1.7414$\pm$0.1497, data are expressed as mean $\pm$ SE) and showed there was no significant difference between STSM and CTSM ($p>0.05$, $n=4$).

Figure 5: MLC_{20} contents in CTSM and STSM

Figure 6. Experimental record of MLC_{20} phosphorylation in ATP-γ-s treated skinned sensitized (A) and control (B) tracheal smooth muscle in a single experiment. MLC: non-phosphorylated 20KDa myosin light chain. MLC-p: mono-phosphorylated 20KDa myosin light chain. MLC-pp: di-phosphorylated 20KDa myosin light chain. Both in sensitized and control groups, the MLC_{20} was maximally phosphorylated (nearly 100%) at about 5 minutes after stimulation.

Figure 6: Time course of MLC_{20} phosphorylation record from ATP-γ-s treated skinned CTSM and STSM

Figure 7. Measurement of MLC_{20} phosphorylation of ATP-γ-s treated control (CTSM) and sensitized (STSM) muscles was undertaken to show that maximum phosphorylation (nearly 100%) was complete in 5 minutes. Since measurements of NADH fluorescence change started at 5 minutes, it was not due to ATP utilization for MLC_{20} phosphorylation, but was an index of the energetics of cross-bridge cycling. Means and standard error bars are plotted. Five observations were used in each group.

Figure 7: Time Course of MLC_{20} Phosphorylation from ATP-γ-s Treated CTSM & STSM

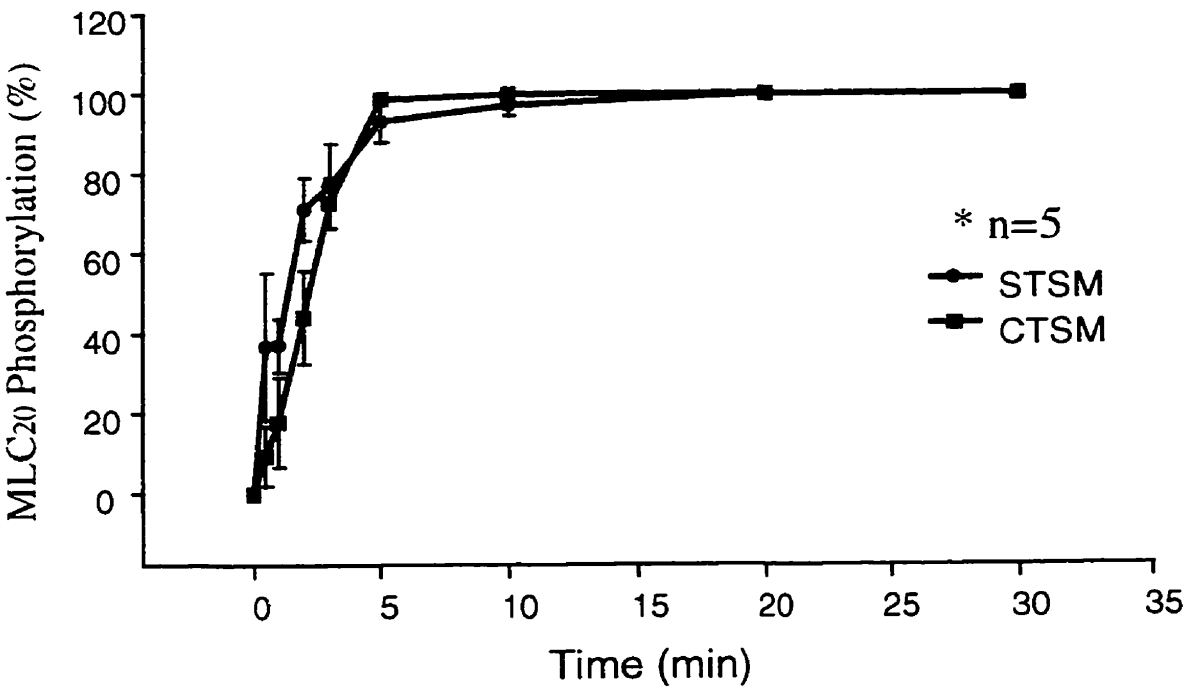

Figure 8. Time course of MLC_{20} phosphorylation (ATP-γ-s was not used) record of skinned CTSM was shown. The upper bands were unphosphorylated 20KDa MLC (MLC); the middle bands were mono-phosphorylated 20KDa MLC (MLC-p); the lower bands were di-phosphorylated 20KDa MLC (MLC-pp). The time course was from 0 to 30 minutes. The ratio of phosphorylated to total MLC_{20} was obtained as the MLC_{20} phosphorylation level.

Figure 8: Time course of MLC_{20} phosphorylation record of skinned CTSM

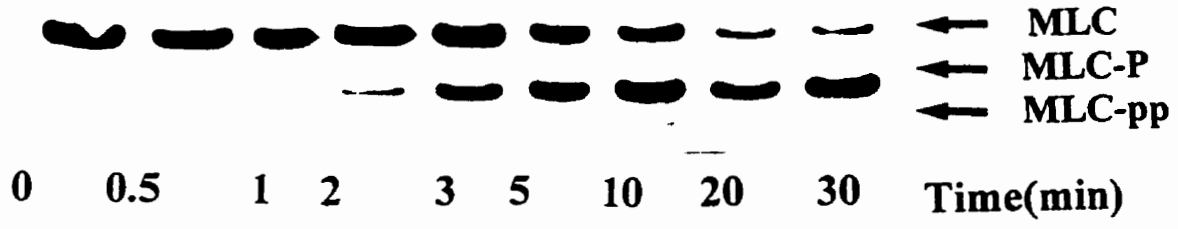

Figure 9. The data obtained from the Figure 8 were plotted to show the time course of MLC_{20} phosphorylation in without ATP-γ-s treated skinned CTSM. It was found that MLC_{20} phosphorylation increased with time course of muscle contraction. It reached the maximal level of ~83% 20 minutes after stimulation with 25 μM Ca^{2+}. Means and standard error bars are plotted. Four observations were used in each group.

Figure 9: Time Course of MLC_{20} Phosphorylation from Skinned CTSM

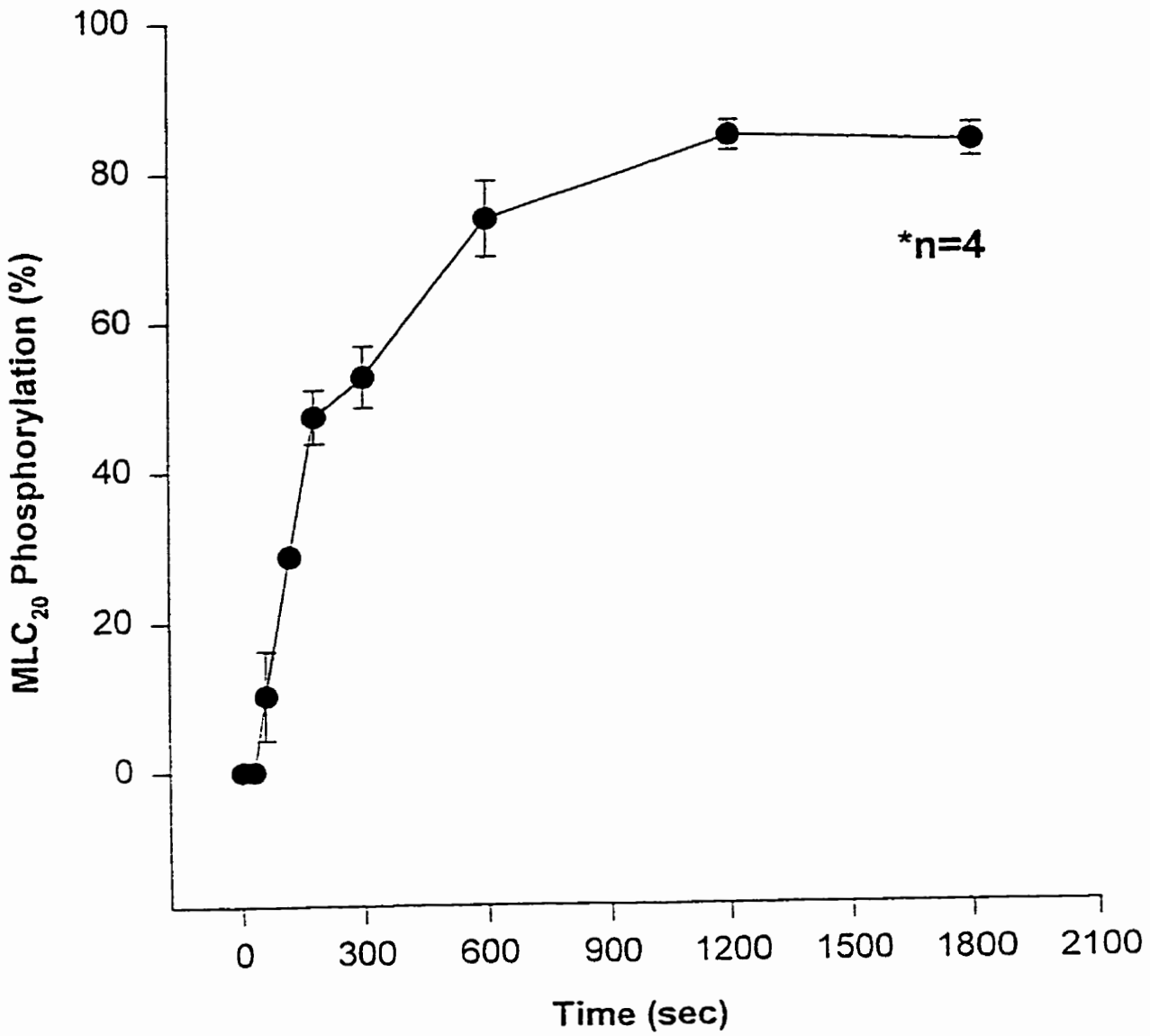

Figure 10. Time course of ATPase activity from ATP-γ-s treated control tracheal smooth muscle (CTSM) (◊) and sensitized tracheal smooth muscle (STSM) (□). The means of seven experiments are shown. Since the 20KDa myosin light chain was thiophosphorylated 5 minutes after stimulation, the time course of ATPase activity was plotted from 200s to 1500s. During this time course, the ATPase activity was not the ATP utilization rate in MLC_{20} phosphorylation, but the ATP utilization rate involved in cross-bridge cycling (actomyosin ATPase activity) only. It is clear that the ATP utilization rate in cross-bridge cycling of STSM is significantly higher than that of the controls ($\alpha<0.05$).

Figure 10: Time Course of ATPase Activity from ATP-γ-s Treated Skinned CTSM & STSM

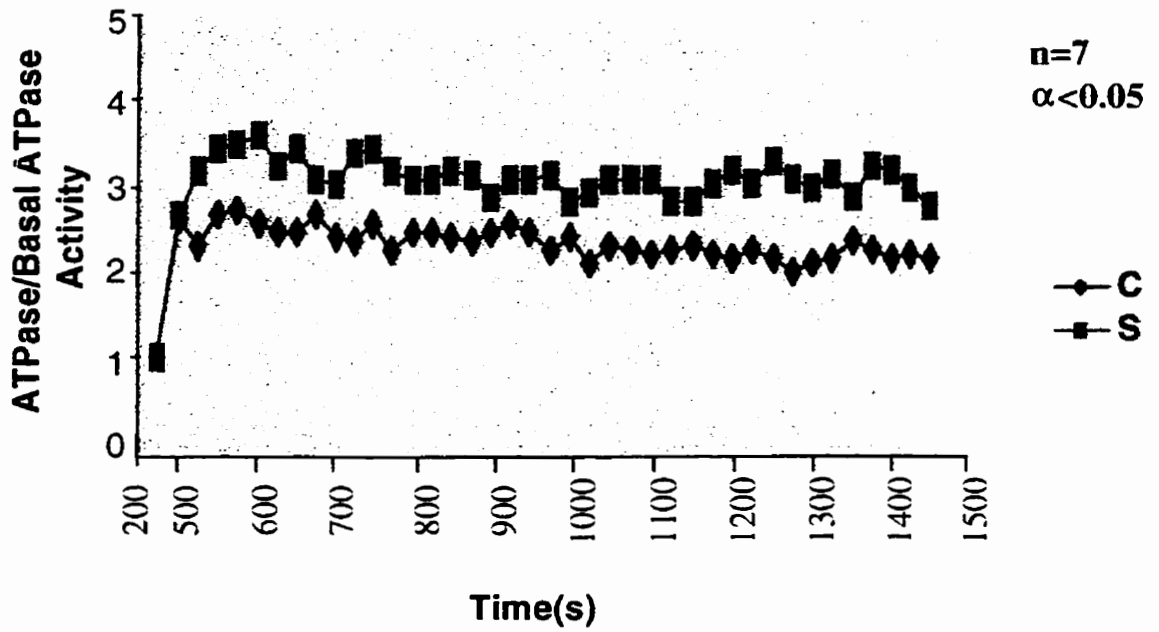

Figure 11. Time course of ATPase activity from CTSM (◊) and STSM (□). The means of seven experiments are shown. Since in these experiments ATP-γ-s was not used, the ATPase activity we obtained here was the total ATPase activity which included the ATP utilization rate by MLC_{20} phosphorylation/dephosphorylation and cross-bridge cycling. The total ATPase activity of STSM was also significantly higher than that of the CTSM ($\alpha<0.05$).

Figure 11: Time Course of ATPase Activity from Skinned CTSM & STSM

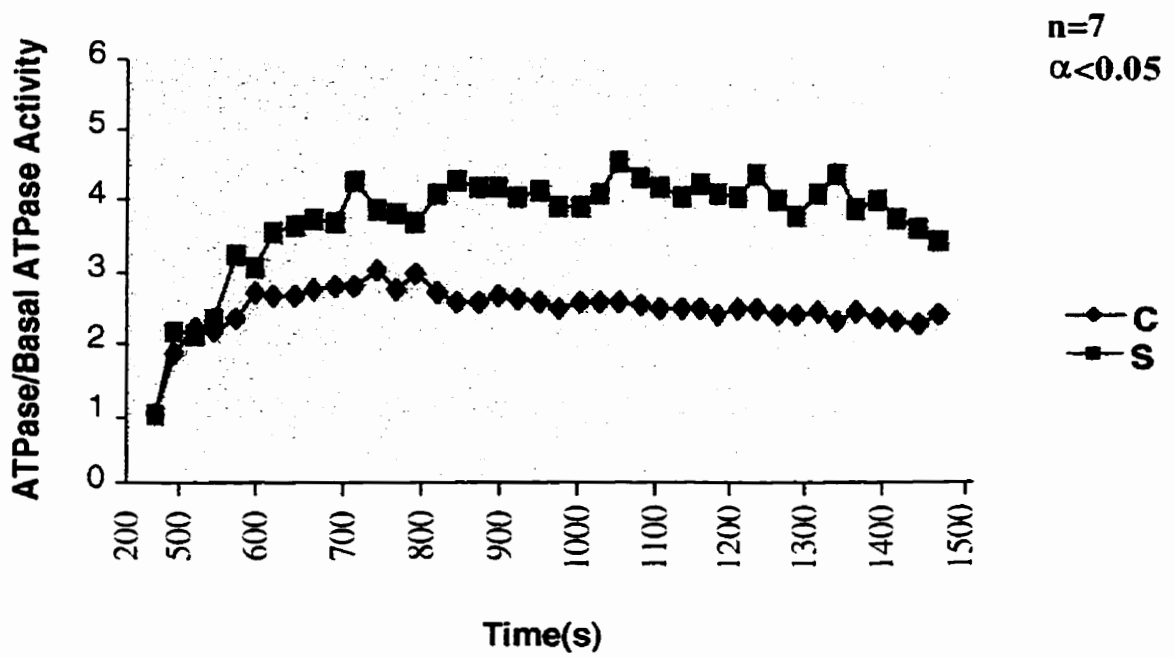

Figure 12. Time course of ATPase activity from CTSM and STSM were compared between with and without ATP-γ-s treated groups. The upper panel is from CTSM and the lower one from STSM. w=with; w/o=without. The means of seven experiments are plotted. In the ATP-γ-s treated group, the ATPase activity was the ATP utilization rate in cross-bridge cycling alone while in the without ATP-γ-s treated group, the ATPase activity was the total ATPase activity ($\alpha<0.05$). We found that both in sensitized and control tracheal smooth muscle, the total ATPase activity was significantly higher than the ATP utilization rate in cross-bridge cycling. The difference between them was mainly due to the ATP utilization in MLC_{20} phosphorylation/dephosphorylation.

Figure 12: Time Course of ATPase Activity

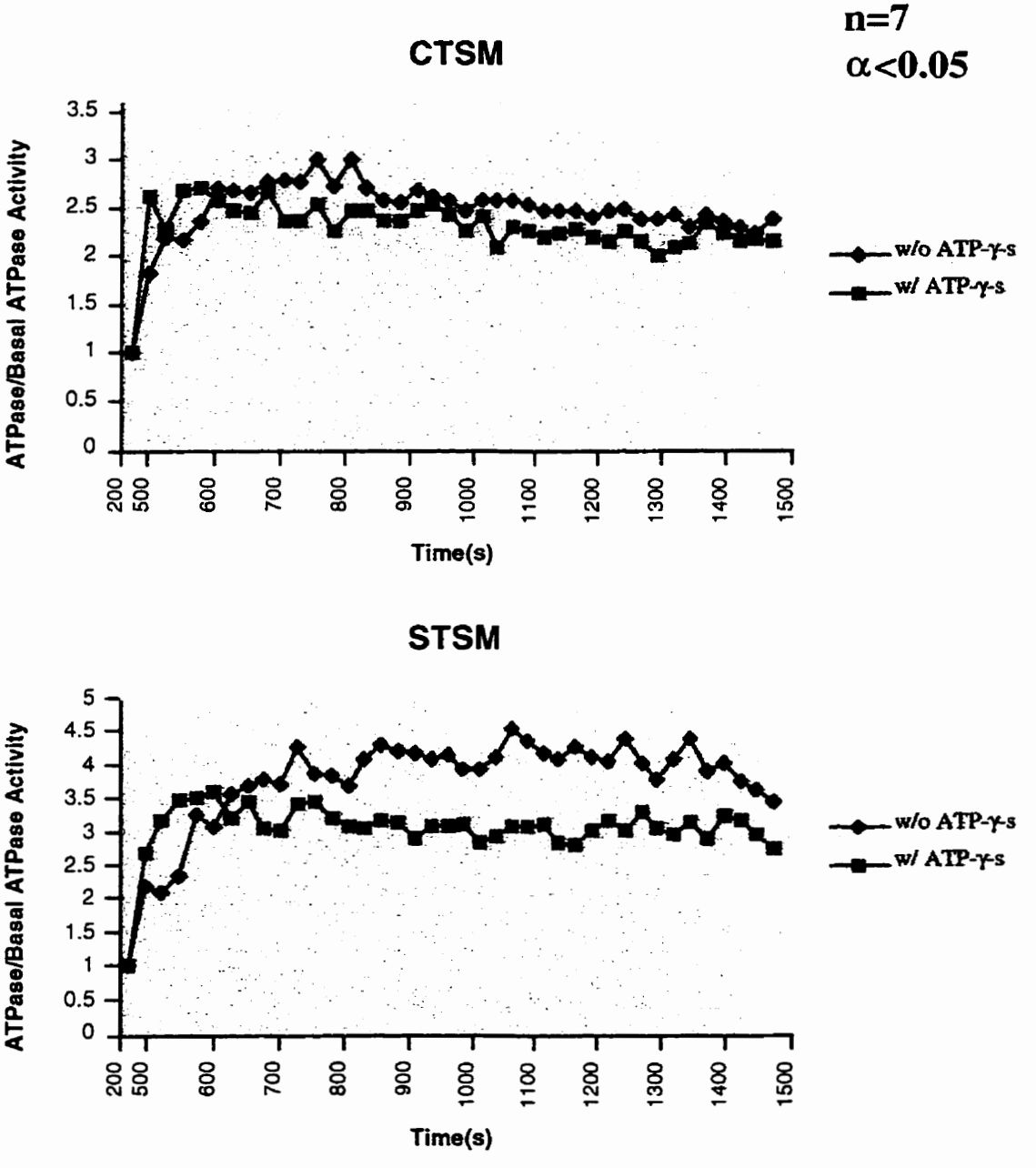

Figure 13. The means analyzed from seven experiments for each curve are shown. The symbols depict the trace obtained from the original records as shown in Figure 4. The box to the right indicates the labeling for the curves shown. C=Control; S=Sensitized; w=with; w/o=without. The blue curve shows the energy utilization rate of both the MLC_{20} phosphorylation and the cycling cross-bridge in sensitized strips. The pink curve represents the energy utilization rate of the cross-bridge alone, since the MLC_{20} has already been fully phosphorylated by ATP-γ-s. Note that the cross-bridge cannot utilize ATP-γ-s. The yellow and black curves are similar curves from muscle strips obtained from littermate control animals. Comparing the sensitized (pink) and control (black) strips it is clear that sensitized cross-bridges utilize more energy for cycling. $\alpha<0.05$ was considered significant.

Figure 13: Time Course of ATPase Activity

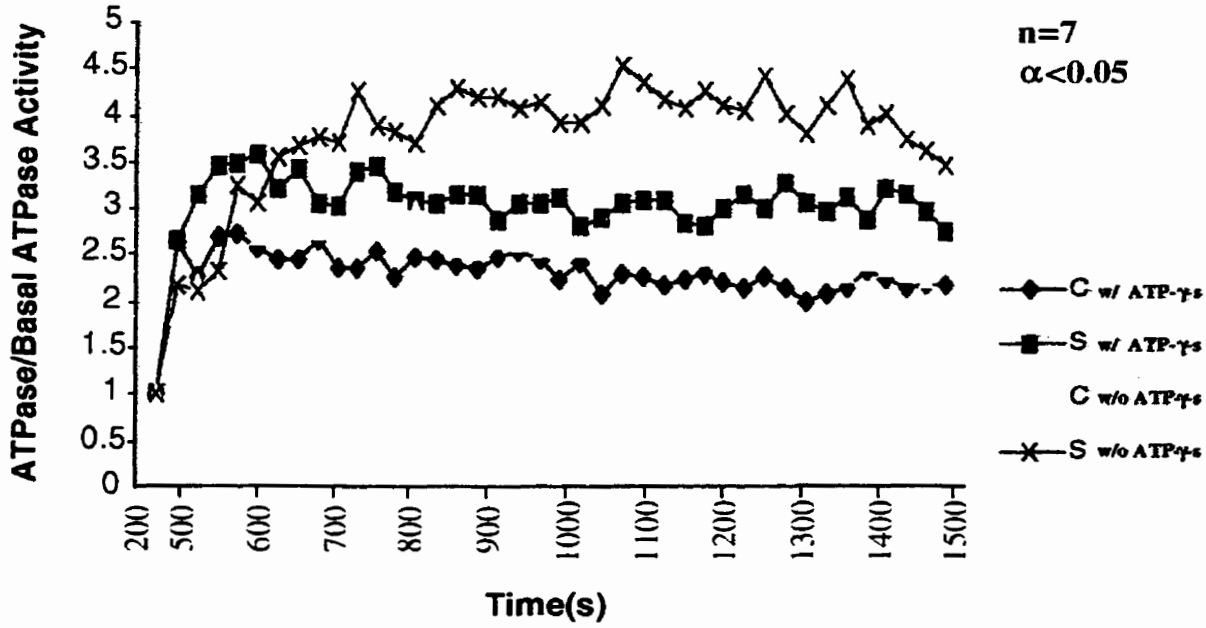

Figure 14. (A) Time course of isometric force (upper trace) and NADH fluorescence signal indicating rate of ATP consumption by the muscle (lower trace). (B) Expansion of the lower trace in (A), illustrating the method for calculating the change in ATP concentration over a period of 25s. Because the signal decline over a 25s period is linear, the amplitude of the "saw-tooth" wave is directly proportional to the ATPase rate. See text for additional description.

Figure 14: Time course of isometric force and NADH fluorescence signal

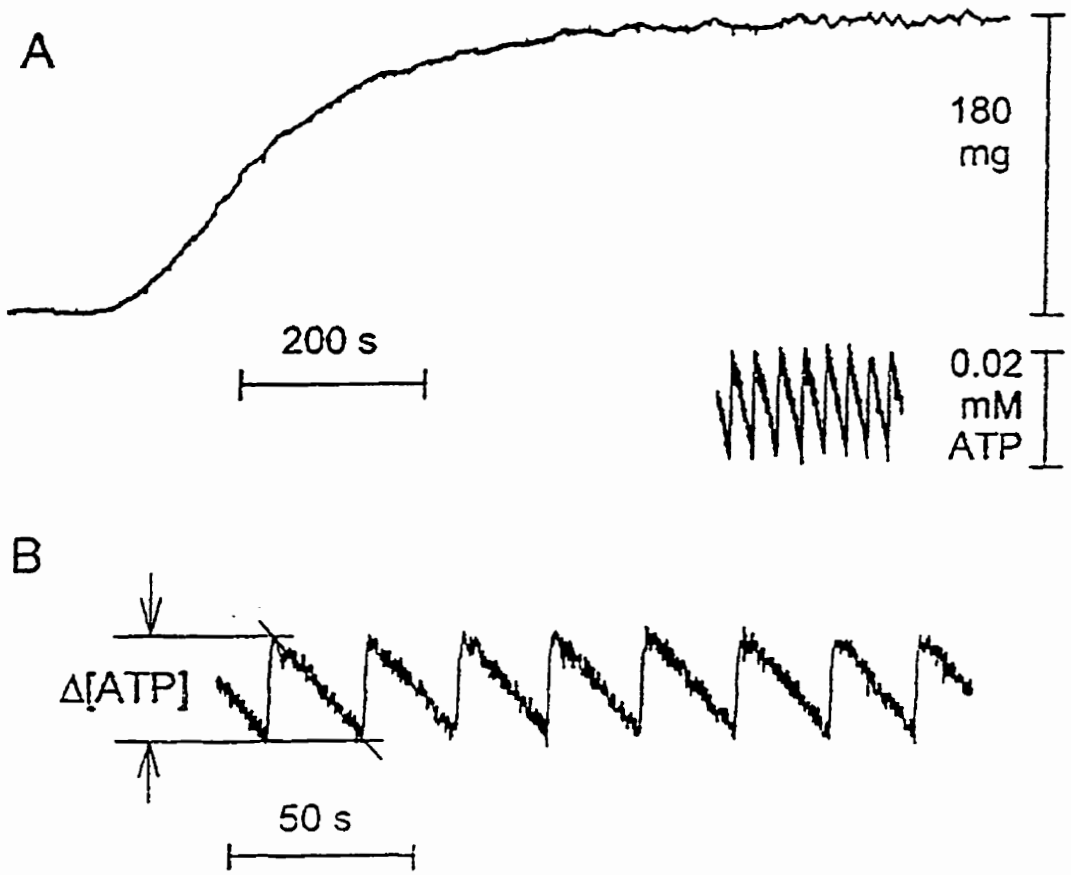

Figure 15. Isometric force (◊) and ATPase rate (O) as functions of muscle length. Muscle lengths are normalized to a reference length (L_{ref}) and both isometric force and ATPase rate are normalized to their respective values at L_{ref}. Means and standard error bars are plotted. Six muscle strips from 2 dogs were used for the experiments.

Figure 15: Isometric force and ATPase rate as functions of muscle length

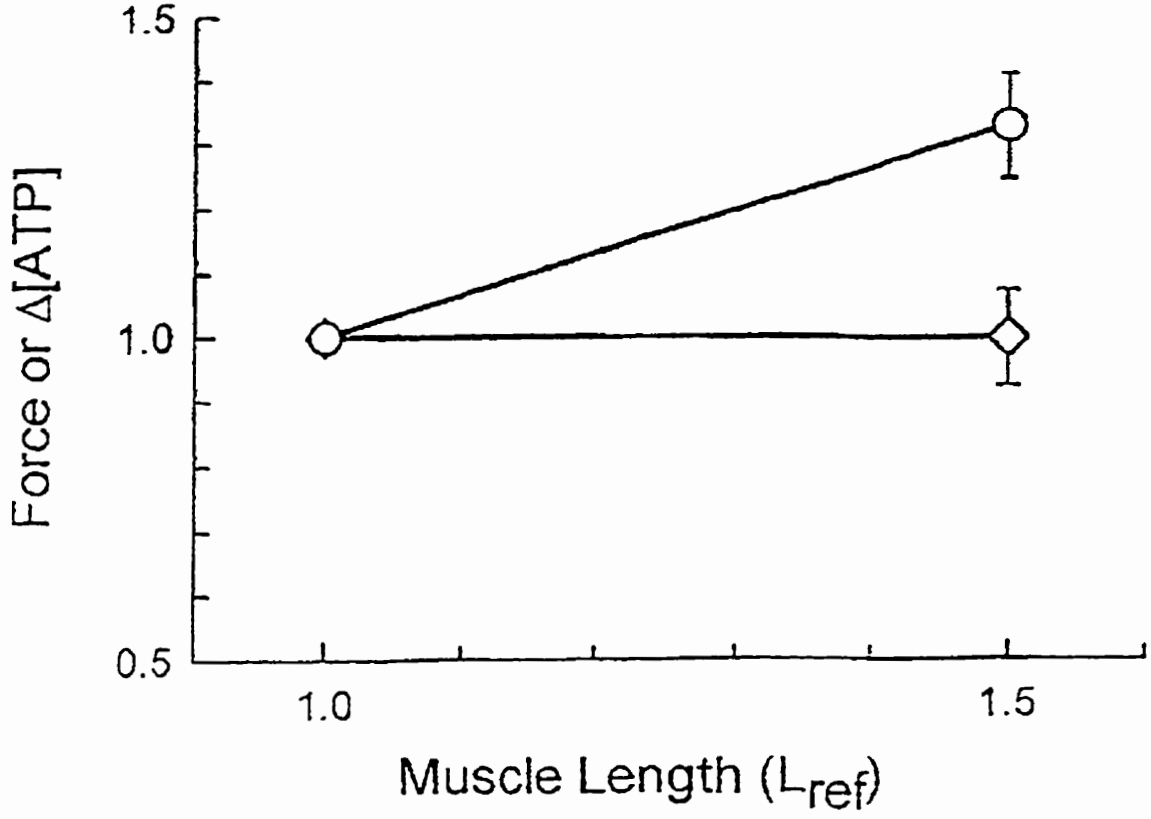

Table 1. Maximum isometric force in skinned CTSM and STSM. Po was normalized to the muscle cross-sectional area. In both with and without ATP-γ-s treated groups, the Pos were the same in sensitized and control tracheal smooth muscles ($p>0.05$).

Table 2. Maximum shortening capacity and velocity in skinned CTSM and STSM. ΔLmax was normalized to %Lo and Vo was normalized to %Lo/sec. Both in the with and without ATP-γ-s treated groups, the ΔLmax and Vo of sensitized TSM were significantly higher than those of the controls ($p<0.05$).

Table 1: Maximum Isometric Tension in Skinned CTSM & STSM

	Without ATP -γ -s		With ATP - γ-s	
	Po - C	Po - S	Po - C'	Po - S'
Mean ± S.E. (mN/mm^2)	170.45 ± 7.06	179.79 ± 24.65	143.16 ± 12.54	150.04 ± 4.84
t-test (P value)	P>0.05		P>0.05	

Table 2: Maximum Shortening Capacity and Velocity in Skinned CTSM & STSM

Without ATP - γ-s

	L-C	L-S	V-C	V-S
Mean ± S.E.(%)	77.74 ± 0.73	88.72 ± 1.35	31.17 ± 1.24	38.85 ± 1.15
Increase(%)	14.12 ± 1.74		24.63 ± 3.69	
t-test (P value)	<0.001		<0.001	

With ATP - γ-s

	L'-C	L'-S	V'-C	V'-S
Mean ± S.E.(%)	77.80 ± 1.57	85.16 ± 1.36	33.67 ± 0.83	39.79 ± 1.74
Increase(%)	9.46 ± 1.74		18.17 ± 5.18	
t-test (P value)	<0.05		<0.05	

Table 3. Total ATP utilization rate, myosin heavy chain ATPase activity and pseudo-ATPase activity from CTSM and STSM are listed. Arbitrary Unit: Activated ATPase/Basal ATPase activity. Data are expressed as mean ± SE. Pseudo-ATPase activity included myosin light chain kinase and phosphatase activity. Total ATP utilization rate, MHC ATPase activity and pseudo-ATPase activity were all increased in sensitized tracheal smooth muscle.

Table 3: Mean Values of ATPase Activity from Skinned CTSM & STSM

	Total ATP Utilization Rate (Arbitrary Unit)	MHC ATPase Activity (Arbitrary Unit)	MLCK + MLCPP Activity (Arbitrary Unit)
STSM	3.4860 $\pm$0.3228	3.0934 $\pm$0.3042	0.3926 $\pm$0.0828
CTSM	2.5631 $\pm$0.1563	2.3140 $\pm$0.3110	0.2491 $\pm$0.0615
Increase(%)	36.01	33.68	57.61
α	< 0.05	< 0.05	< 0.05

Table 4. Force and ATPase activity at different muscle lengths. Means and standard errors are listed. Paired ratios are ratios of respective values from the same muscle strip. Some of the resting tensions at L_{ref} were zero, therefore the resting force ratio was not calculated. Some of the resting ATPase rates were too small, less than the noise level, therefore only the estimated upper limits were given. *, Statistically different from unity, $p<0.01$.

Table 4: Force and ATPase Activity at Different Muscle Lengths

			Paired Ratio
Muscle Length	3.32 ± 0.13	4.96 ± 0.18	1.5 ± 0.0
Active Force (kPa)	68.7 ± 5.4	69.2 ± 9.3	0.998 ± 0.074
Resting Force (kPa)	1.66 ± 0.84	39.4 ± 7.3	--
Active ATPase Rate (μM/s)	1.17 ± 0.42	1.49 ± 0.47	1.327 ± 0.082 *
Resting ATPase Rate (μM/s)	< 0.15	< 0.17	--

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IMAGE EVALUATION
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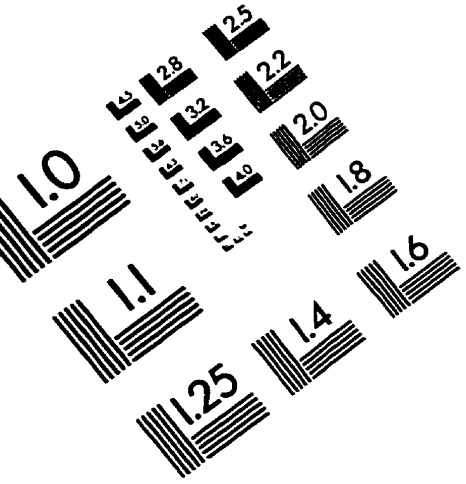

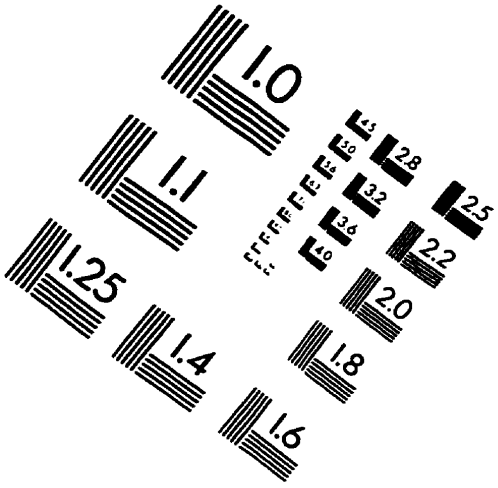

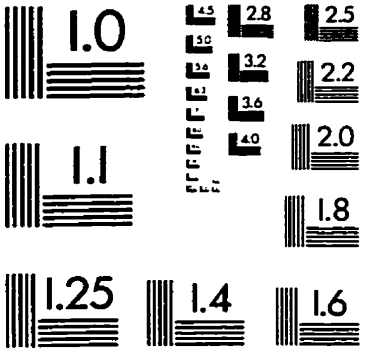

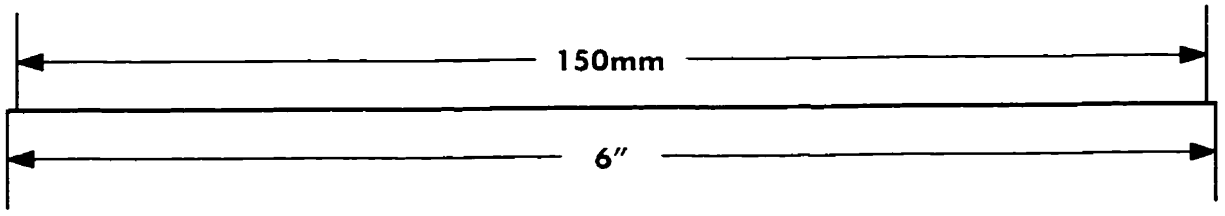

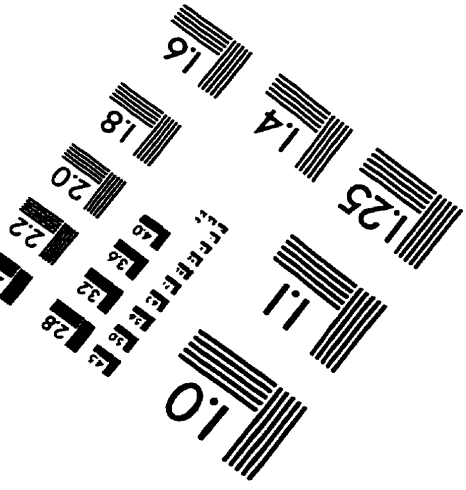

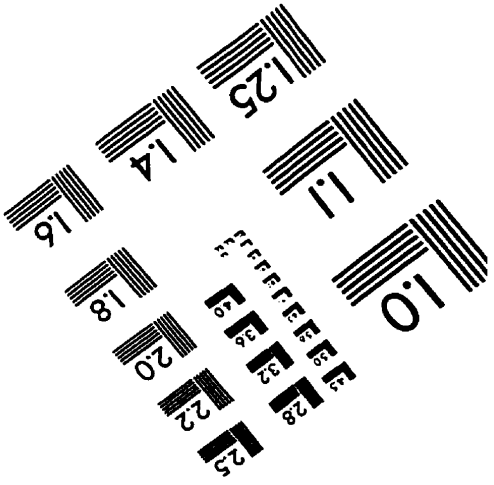

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