Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects

Xuefei Ma, Zhaoqin Cheng, Hong Kong, Ying Wang, Helmut Unruh, Newman L. Stephens, and Michel Laviolette. Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. Am J Physiol Lung Cell Mol Physiol 283: L1181–L1189, 2002. First published June 27, 2002; 10.1152/ajplung.00389.2001.—Whether contractility of bronchial smooth muscle cells (BSMC) from asthmatic subjects is significantly altered has never been validated. We tested the hypothesis that such BSMC show increased contractility. Cells were isolated from endobronchial biopsies. BSMC shortening was measured under an inverted microscope. Statistically significant increases in maximum shortening capacity (ΔL_{max}) and velocity (V_o) were found in asthmatic BSMC compared with normal cells. Mean ΔL_{max} in asthmatic BSMC was 39.05 ± 1.99% (SE) of resting cell length compared with 28.6 ± 1.1% in normal cells; mean V_o was 7.2 ± 0.8% of resting cell length/s in asthmatic cells and 5.23 ± 0.46% in normal cells. To investigate the mechanism of the increased contractility, we measured mRNA abundance of smooth muscle types of myosin light chain kinase (smMLCK) and myosin heavy chain. RT-PCR data revealed that smMLCK mRNA was higher in asthmatic BSMC (0.106 ± 0.021 arbitrary densitometric units, n = 7) than in control cells (0.04 ± 0.008, n = 11; P < 0.05). Messages for myosin heavy chain isoforms showed no difference. Increased kinase message content is an index of the mechanism for the increased velocity and capacity of shortening we report.

asthmatic bronchial smooth muscle contractility; smooth muscle myosin light chain kinase; single bronchial smooth muscle cell mechanics; single bronchial smooth muscle cell biochemistry

The high prevalence and increasing mortality of asthma have focused research on the elucidation of its pathogenesis. Consensus in the field is that asthma is primarily a chronic inflammatory disease. Nevertheless the fact that β2-adrenergic agonists can relieve the respiratory distress of the acute asthmatic attack and ameliorate that of chronic asthma suggests that besides airway inflammation, contraction of airway smooth muscle plays an important role in asthma pathophysiology. Whether the contractile properties of human asthmatic airway smooth muscle are altered has never been demonstrated apart from anecdotal reports in which tissues were obtained postmortem or surgically (4, 6). The argument that the bronchial smooth muscle cells (BSMC) are the seat of primary changes in the mechanical properties of the asthmatic airways is supported by findings of increased maximum capacity (ΔL_{max}) and velocity (V_o) of shortening in BSMC obtained from our canine model of ragweed pollen-induced allergic hyperreactivity (10). Airway smooth muscles from these animals showed no evidence of inflammation. In human asthma, bronchospasm has been reported in the absence of acute inflammation in exercise-induced asthma, where bronchoalveolar lavage revealed no significant elevation in levels of inflammatory mediators and/or cells (5). Studying the immediate and late asthmatic response, Liu et al. (17) reported that the 5-min bronchoconstrictor response to antigen challenge was not associated with increases in bronchoalveolar lavage cell number nor in airway permeability, indicating that airway smooth muscle contraction plays a predominant role in the immediate asthmatic response. On the other hand, airway inflammation has been found in cases of very mild asthma and also in subjects with increased airway reactivity but no asthma symptoms (14, 15). In this latter group, the airway inflammation increased over a 2-yr period and was more important in those who developed symptoms of asthma (15). These observations suggested that, in human asthma, there is an asymptomatic period in which inflammation induces airway hyperreactivity; when this process intensifies, symptoms appear. Consequently, although its relation with airway inflammation is not well defined, airway contractility is likely a major component of asthma pathogenesis, and, in the present study, we postulated that BSMC from asthmatics show increased contractility compared with cells from normal subjects.

We have previously reported that airway smooth muscle myofibrillar Mg2+-ATPase activity is increased

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in ragweed pollen-sensitized dogs compared with un-sensitized animals (13). In that study, sensitized dog airway smooth muscles presented no increase in myosin heavy chain (SM-A) content that includes myosin heavy chain-1 (SM-1) and -2 (SM-2) isoforms; increased total content of smooth muscle myosin light chain kinase (smMLCK) was detected. This kinase was of the smooth muscle type with a molecular mass of 108 kDa (smMLCK-108). It stimulated actin-activated myosin Mg$^{2+}$-ATPase activity via phosphorylation of the 20-kDa myosin light chain (MLC20). As expected, these smooth muscles also presented an increased phosphorylation of the regulatory MLC20 (13). Consequently, SM-A, and SM-B. This study provides evidence of an increased contractility in human asthmatic BSMC and indicates that this is likely mediated by increased cell contractility.

The specific aims of this project were to measure the $\Delta L_{\text{max}}$ and $V_o$ of isolated BSMC obtained from endobronchial biopsy samples from asthmatics and controls and to investigate the underlying molecular mechanisms by measuring the abundance of mRNA for smMLCK-108, SM-A, and SM-B. This study provides statistically significant evidence of an increased contractility in human asthmatic BSMC and indicates that this is likely mediated by increased total activity and content of smMLCK-108.

**MATERIALS AND METHODS**

Selection and evaluation of subjects. The mechanical and biochemical studies were conducted on endobronchial biopsies from two subpopulations randomly selected from a defined, homogeneous parent population of mild to moderately severe asthmatics whose ages (with two exceptions aged 44 and 48 yr) ranged from 18 to 28 yr. The paucity of BSMC nonasthmatics and asthmatic subjects had upper or lower airway infections during the month preceding the bronchoscopy. All were current nonsmokers. Three asthmatic subjects had smoked $>$7 packs/yr and quit $>$4 yr ago. The evaluation included a medical history, a physical examination, skin prick tests to common allergens (animal dander, house dust mites, pollens, grass, and molds; these were obtained from the American Thoracic Society (1, 2). With two exceptions that disease, no occupational exposure to sensitizing agents, having no history of asthma nor of taking any medication for that disease, no occupational exposure to sensitizing agents, and no history of disease, except for numbers 6–11, who suffered from allergic rhinitis (Table 2). In asthmatic subjects, the diagnosis was made according to the definition of the American Thoracic Society (1, 2). With two exceptions who were on steroids, only subjects who were taking inhaled $\beta_2$-agonist alone, on demand, were included in the study. No other asthma drug was being used at the time. None of nonasthmatic and asthmatic subjects had upper or lower airway infections during the month preceding the bronchoscopy. All were current nonsmokers. Three asthmatic subjects had smoked $>$7 packs/yr and quit $>$4 yr ago. The evaluation included a medical history, a physical examination, skin prick tests to common allergens (animal dander, house dust mites, pollens, grass, and molds; these were obtained from Omega, Montreal, Canada), a spirometry (2), and measurement of airway responsiveness to methacholine according to standardized procedures (1). Concentrations of methacholine up to 256 mg/ml were used, and the responses were expressed as the provocative concentration of methacholine inducing a 20% fall in forced expiratory volume in 1 s (FEV$_1$) in asthmatic subjects via a metered-dose inhaler (Glaxo Wellcome, Mississauga, Canada) when their FEV$_1$ was $<$75% of predicted. Oxygen was administered, and vital signs, electrocardiogram, and oximetry were monitored throughout the endoscopy. After local anesthesia of the throat, larynx, and trachea with 4% lidocaine, a flexible bronchoscope (Olympus OES 10 fiberscope; Olympus, Markham, Canada) was introduced into the bronchial tree. Bronchial anesthesia was completed with lidocaine up to a total dose of 400 mg using either a 2 or 4% solution of lidocaine. Six to eight specimens were taken from the origins of segmental or lobar carinae of the right lung using alligator forceps. After the bronchoscopy, inhaled salbutamol was administered if necessary and subjects were kept under observation until it was felt they could safely resume normal activity. For evaluation of BSMC contractility, the bronchial specimens were suspended in modified low-calcium Krebs solution, pH 7.4, and kept on ice until processed. They were sent by plane to Winnipeg for contractility studies. In all cases the entire procedure was completed within 24 h. For mRNA measurements, bronchial biopsies

<table>
<thead>
<tr>
<th>Sex</th>
<th>FVC, l</th>
<th>FEV$_1$, l</th>
<th>PC$_{20}$, mg/ml</th>
<th>Allergy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonasthmatics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>5.54(112)</td>
<td>5.29(126)</td>
<td>128</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>4.46(144)</td>
<td>6.74(148)</td>
<td>256</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>4.34(113)</td>
<td>3.88(119)</td>
<td>256</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>5.08(94)</td>
<td>4.47 (96)</td>
<td>38.6</td>
<td>none</td>
</tr>
<tr>
<td>M</td>
<td>7.17(128)</td>
<td>5.20(111)</td>
<td>$&gt;$128</td>
<td>none</td>
</tr>
<tr>
<td>Asthmatics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>3.92(100)</td>
<td>3.39(101)</td>
<td>2.57</td>
<td>Du, DPt</td>
</tr>
<tr>
<td>M</td>
<td>3.45(70)</td>
<td>2.17(56)</td>
<td>1.8*</td>
<td>C, D, Rw, Du</td>
</tr>
<tr>
<td>F</td>
<td>3.25(78)</td>
<td>2.10(66)</td>
<td>1.8*</td>
<td>C, D, Rw</td>
</tr>
<tr>
<td>M</td>
<td>3.22(89)</td>
<td>1.92(64)</td>
<td>4.0*</td>
<td>C, D, Du</td>
</tr>
<tr>
<td>F</td>
<td>3.95(112)</td>
<td>4.32(96)</td>
<td>0.53</td>
<td>C, D, Du, M</td>
</tr>
</tbody>
</table>

M, male; F, female. Forced vital capacity (FVC) and prebronchodilator forced expiratory volume in 1 s (FEV$_1$) are expressed in liters and (%) of predicted. PC$_{20}$, provocative concentration of methacholine inducing a 20% fall in FEV$_1$. Allergy was defined as mean wheal diameter of skin response read at 10 min if $\leq$3 mm. Rw, ragweed; D, dog; C, cat; Du, dust; M, mold; DPt, D. Pterosynnus. A wheal response to histamine $\geq$3 mm served as a positive control. For statistical analysis, presence of allergy was designated as 1, whereas its absence was designated as 0.
were rapidly frozen and sent, on dry ice, to Winnipeg. On arrival they were thawed to 22°C in normal Ca\textsuperscript{2+} (1.6 mM) containing Krebs-Henseleit solution and bubbled with a 5% CO\textsubscript{2}-95% air gas mixture.

**Preparation of contractile bronchial smooth muscle cells.** From each subject, all bronchial samples were pooled and incubated in Hanks’ solution with 10 mM taurine, 400 units/ml collagenase, 30 units/ml papain, 1 mg/ml BSA, and 1 mM DTT for 45 min. After enzymatic digestion, the tissue units/ml collagenase, 30 units/ml papain, 1 mg/ml BSA, and by 25 determined by light microscopy, and their ability to shorten ability to exclude trypan blue, their typical fusiform shape five percent of the cells isolated were viable as judged by their sarcomere, which could be clearly recognized by the presence of membranous blebs pouching out from the sarcolemma. Shortening capacity of single cells was measured by applying a 10-Hz, 35-V, 1-ms-long bipolar electric pulse stimulation, which could be clearly recognized by the presence of membranous blebs pouching out from the sarcolemma. Shortening capacity of single cells was measured by applying a 10-Hz, 35-V, 1-ms-long bipolar electric pulse stimulation, with one spot electrode placed ~10 μm away from the cell and the other at a random position in the chamber. This single pulse elicited a shortening response that was 85% of the maximal response to acetylcholine (10\textsuperscript{-4} M). The shortening response to the single electrical pulse was fully reversible as stated above. Stimulation with 10\textsuperscript{-4} M acetylcholine produced a contraction 20% greater than that elicited by EFS, 80 mM KCl produced a contraction almost equal to that of EFS. However, these contractions were not fully reversible and were not used in subsequent experiments. Throughout the experiment, images of the cells were monitored and recorded by a closed-coupled device video camera mounted on the microscope. The images were then digitized and analyzed with an in-house-designed computer program for derivation of \(\Delta L_{\text{max}}\) and \(V_{o}\), \(V_{o}\) represents zero-loaded isotonic shortening. To obtain this measurement, the cell was gently lifted off the bottom of the glass chamber. One end was held in a glass microcath while the other end remained free. The entire cell was held under the surface of the perfusing solution but

Table 2. Characteristics of subjects: RT-PCR experiments

<table>
<thead>
<tr>
<th>Subject Numbers</th>
<th>Age, yr</th>
<th>Sex</th>
<th>FVC, l</th>
<th>FEV\textsubscript{1}, l</th>
<th>PC\textsubscript{20}, mg/ml</th>
<th>Allergy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nonasthmatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>28(DM)</td>
<td>M</td>
<td>5.54(122)</td>
<td>5.29(126)</td>
<td>128</td>
<td>none</td>
</tr>
<tr>
<td>12</td>
<td>27(NP)</td>
<td>F</td>
<td>4.81(122)</td>
<td>4.03(109)</td>
<td>128</td>
<td>none</td>
</tr>
<tr>
<td>13</td>
<td>29(RD)</td>
<td>M</td>
<td>7.19(129)</td>
<td>5.18(113)</td>
<td>128</td>
<td>none</td>
</tr>
<tr>
<td>14</td>
<td>25(MR)</td>
<td>F</td>
<td>3.90(98)</td>
<td>3.57(104)</td>
<td>128</td>
<td>none</td>
</tr>
<tr>
<td>15</td>
<td>28(SR)</td>
<td>F</td>
<td>4.23(108)</td>
<td>3.38(110)</td>
<td>55</td>
<td>none</td>
</tr>
<tr>
<td>16</td>
<td>22(PF)</td>
<td>F</td>
<td>4.36(110)</td>
<td>3.31(103)</td>
<td>14.18</td>
<td>T, G</td>
</tr>
<tr>
<td>17</td>
<td>22(SHC)</td>
<td>F</td>
<td>3.56(92)</td>
<td>3.15(101)</td>
<td>36.5</td>
<td>T, Du, Dp, Df</td>
</tr>
<tr>
<td>18</td>
<td>44(JL)</td>
<td>M</td>
<td>4.72(89)</td>
<td>3.75(95)</td>
<td>19.56</td>
<td>C, G, H, Dpt, Df</td>
</tr>
<tr>
<td>19</td>
<td>25(JFB)</td>
<td>M</td>
<td>5.08(104)</td>
<td>4.30(108)</td>
<td>98.54</td>
<td>Rw, T, C, D, H, DpT</td>
</tr>
<tr>
<td>20</td>
<td>25(BH)</td>
<td>M</td>
<td>5.08(93)</td>
<td>3.86(84)</td>
<td>119.9</td>
<td>C, D, H, Dpt, Df</td>
</tr>
<tr>
<td>21</td>
<td>22(SB)</td>
<td>F</td>
<td>3.79(100)</td>
<td>3.47(114)</td>
<td>83.6</td>
<td>G, Rw, T, Dpt</td>
</tr>
<tr>
<td><strong>Asthmatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>22(FB)</td>
<td>M</td>
<td>6.68(122)</td>
<td>5.10(107)</td>
<td>0.26</td>
<td>Rw, C, Du</td>
</tr>
<tr>
<td>23</td>
<td>21(CM)</td>
<td>F</td>
<td>3.83(99)</td>
<td>3.18(96)</td>
<td>5.8</td>
<td>Rw, C, D, Du, H</td>
</tr>
<tr>
<td>24</td>
<td>21(MED)</td>
<td>M</td>
<td>4.65(117)</td>
<td>3.06(90)</td>
<td>1.05</td>
<td>Du, D, Dpt, Rw</td>
</tr>
<tr>
<td>25</td>
<td>18(MC)</td>
<td>M</td>
<td>4.69(98)</td>
<td>3.96(96)</td>
<td>1.3</td>
<td>T, Du, G</td>
</tr>
<tr>
<td>26</td>
<td>48(MDL)</td>
<td>F</td>
<td>3.12(90)</td>
<td>2.29(81)</td>
<td>0.17</td>
<td>Rw, C, Du, D</td>
</tr>
<tr>
<td>27</td>
<td>22(YC)</td>
<td>M</td>
<td>4.88(115)</td>
<td>3.72(102)</td>
<td>2.82</td>
<td>none</td>
</tr>
<tr>
<td>28</td>
<td>20(RC)</td>
<td>F</td>
<td>3.99(102)</td>
<td>3.01(93)</td>
<td>Fall of FEV\textsubscript{1} to 65%</td>
<td>none</td>
</tr>
</tbody>
</table>

Subject’s age is given in years with his or her initials in parentheses.
was not allowed to touch the glass surface. $V_o$ was measured from the digitized shortening vs. time curve. As is usual in the field, the slope of the curve at 100 ms after onset of shortening was identified as $V_o$. It is to be noted that from a given patient 10–20 airway smooth muscle cells were isolated. These contracted reversibly to EFS, which was the basis for their selection for measurement of velocity ($V_o$) and capacity ($\Delta L_{max}$) of shortening. Nonmuscle cells, e.g., epithelial, lymphocyte, fibroblasts, and macrophages, shortened at most by 5% of their initial length. Moreover, the shortening is irreversible.

Measurement of mRNA of smMLCK-108 and myosin heavy chain (SM-A and SM-B): isolation of total mRNA. From the endobronchial biopsies obtained from subjects described in Table 2 and using the method described above, we isolated BSMC and viewed them under the microscope. Their fusiform morphology and their shortening response to electrical stimulation, as stated before, identified them. The cells obtained from a given asthmatic or control subject were homogenized in 75 $\mu$l of TRIzol reagent. To this was added 75 $\mu$l of chloroform; the mixture was shaken well and then centrifuged. The aqueous phase was collected, isopropanol was added, and the resulting mixture was spun down. The pellet obtained was washed with ethanol, dried, and then brought into solution with 4 $\mu$l of diethylpyrocarbonate-treated water. An alternate procedure was to simply homogenize all the biopsy samples obtained from a given patient. The mRNA from the homogenate total RNA was isolated, and RT-PCR was carried out as described below. The rationale for this procedure was that only smMLCK-108 mRNA was found to be amplified in pilot experiments. Because this message is present only in mature smooth muscle cells, contamination from nonmuscle cells such as epithelial cells, fibroblasts, and macrophages was ignored as these contain nonmuscle-type MLCK (nmMLCK-210) alone, which has low specific activity.

RT-PCR. One microliter of oligo dT (100 ng/μl) was added to 9 μl of the above mRNA solution and incubated at 70°C for 5 min; a further 5-min incubation was conducted under ice-cold conditions. To this was added the following solution: 10 μl 5 X RT buffer (GIBCO), 5 μl 0.1 M DTT, 4 μl 10 mM dNTP, 1 μl RNase inhibitor, 17.5 μl sterile water, 2.5 μl 200 U/μl Moloney murine leukemia reverse transcriptase (M-MLV-RT, GIBCO). This total volume was mixed well and then incubated at 37°C for an hour and a half. Thereafter the tube was placed in 95°C water for 5 min to stop the reaction. Five microliters of the reaction solution were taken for the PCR. The smMLCK-108 mRNA primers were as follows: upstream: 5'-GAATTAGATCTGAGGAATTTCCAGACG-3' and downstream: 5'-CAATTTCTGGATCTTCTTCAATAGGGC-3'. We used 50 μl of PCR reaction solution. It contained 10 μl 5 X PCR buffer (Boehringer Mannheim, Quebec, Canada), 4 μl 10 mM dNTP, 1 μl upstream primer, 1 μl downstream primer, and 1 μl enzyme mixture (Boehringer Mannheim). Sterile water was added to make a final volume of 50 μl and then mixed carefully. According to the thermocycler temperature protocol, reaction solution was heated at 95°C for 2 min to obtain a hot start; 90°C denaturation was carried out for 30 s, 55°C annealing for 30 s, and 72°C for 45 s, and primer extension for 40 cycles followed by incubation for 7 min at 68°C to provide adequate elongation. Dr. Ian Dixon, a departmental colleague, kindly synthesized the primers. The procedure just described was also carried out for myosin heavy chain, along with GAPDH analysis (19). The myosin heavy chain message primers were as follows: upstream: 5'-GT-CATCCAGTACCCTGCGGCG-3' and downstream: 5'-GTCATT-GACCGTCTTTG-3'. The primers for GAPDH were: upstream: 5'-TGAAGGTCCGGTCAACAGAT-3' and downstream: 5'-CATGGAGGCATGAGTCCACCAC-3'. The products of the PCR were separated on 1.5% agarose gel and visualized by staining with ethidium bromide under ultraviolet light. The primers were a generous gift from Drs. R. S. Adelstein and S. Kelley of the National Institutes of Health (Bethesda, MD).

Data analysis. Data were expressed as means ± SE. Cell shortening velocity and capacity were expressed in normalized units with respect to initial cell length. Analysis of variance (ANOVA) was employed to analyze the differences of mean values among different groups. Duncan’s new multiple-range test (8) was used as a complementary analysis to the ANOVA to determine which differences between means were significant ($P < 0.05$). The unpaired two-tailed Student’s $t$-test (7) was used to compare mean values between two groups as needed. Two different populations of normal and asthmatic subjects were used to obtain biopsies for the mechanical and the RT-PCR. The two samples of asthmatics and of nonasthmatics were obtained randomly. Nonparametric statistical analysis (Wilcoxon’s test) was carried out to compare the two subgroups of nonasthmatic subjects and the two subgroups of asthmatic subjects.

RESULTS

Subjects’ characteristics. Subjects’ characteristics are presented in Tables 1 and 2. Nonasthmatic nonallergic ($n = 9$) and allergic ($n = 6$) subjects had normal spirometry parameters and PC$_{20}$ values. Ten asthmatics had FEV$_1 > 75\%$ of predicted and were using β2-agonists alone occasionally. Their PC$_{20}$ was measured at the time of evaluation. Two others had mildly uncontrolled asthma. They were taking inhaled β2-agonist alone on demand at the time of the bronchoscopy, but their PC$_{20}$ was measured after the bronchoscopy and a regular use of inhaled steroids. It was not possible to conduct both sets of measurements on cells obtained from the same subjects, as their number (10–20 cells) was too small, and all biopsies were used for BSMC isolation. In the first group of subjects (Table 1), only mechanical studies were conducted; in the second group (Table 2), only RT-PCR studies were conducted. Nonparametric (Wilcoxon’s two-sample test) analysis showed no significant difference ($P > 0.05$) among the relevant variables (age, sex, forced vital capacity percent, FEV$_1\%$, PC$_{20}$) in the two nonasthmatic groups and the two asthmatic groups.

Contractile properties of human airway smooth muscle cells. As stated above, usually 10–20 contractile cells were isolated from the total number of bronchoscopy biopsies obtained from each subject. The means for asthmatics and controls were $15 ± 3.1$ (SE) and $12 ± 2.9$, respectively. This represented ~5% of the total number of cells in 0.5 mm$^2$ of bronchial airway wall tissue. This figure was obtained by morphometry of a histological tissue section. Freshly isolated BSMC appeared elongated with smooth and shining sarcolemma under an inverted phase-contrast microscope. These cells showed a reversible contraction in response to low-level single-pulse EFS. Maximal contraction could be induced with repeated-pulse electrical stimulation. It was ~15% greater than the response to a single pulse but was irreversible. BSMC demonstrated
a mean length of 70 ± 5 μm at full relaxation for both asthmatic and control subjects.

Figure 1A shows isotonic shortening of an essentially zero-loaded, electrically stimulated single human BSMC from a normal subject, measured at room temperature (22°C). At 4 s, shortening is 50% complete. At 37°C (data not shown), the shortening is almost equal to 75% at 4 s and equal to that of canine BSM strips previously reported by us (see Fig. 6 in Ref. 23). Initial experiments on human BSMC conducted at 37°C revealed deterioration of cell condition. At 22°C stability was very good. For this reason and for the fact that the biochemical studies were conducted at 22°C, this was the temperature selected for the experiments. From data such as this, contractility as assessed by measuring ΔLmax and V0 was compared between five asthmatic and five nonasthmatic subjects. A significant difference was identified in contractile properties between asthmatic and nonasthmatic BSMC (Fig. 1B). The former showed increased V0 and ΔLmax of unloaded shortening compared with those of the latter. In asthmatic BSMC, ΔLmax was 39.05 ± 1.99% of resting cell length and V0 was 7.2 ± 0.8% of resting cell length per s, whereas in the control cells they were 28.6 ± 1.1% and 4.75 ± 0.76%, respectively (P < 0.05). The magnitude of shortening seen in these cells proves they were smooth muscle cells. All other cell types present in the biopsies, such as fibroblasts, epithelial cells, leukocytes, and macrophages, showed only 5% or less shortening, which was moreover irreversible.

Molecular mechanism for the increased shortening of asthmatic airway smooth muscle. To investigate the molecular mechanism for the increased V0 that we report here, we measured the abundance of the messenger RNA for smMLCK-108. Figure 2 shows results for smMLCK-108 mRNA. Lane 1 shows molecular weight markers, lanes 2–7 show bands from asthmatic subjects, whereas bands 8–10 are from nonasthmatics. The average density of the bands in lanes 2–7 is greater than that of the average density of bands in lanes 8 and 10. Similar results were obtained with the other asthmatic and the two normal subjects. We conclude that the content of mRNA for smMLCK-108 is significantly greater in asthmatics. The mean values expressed in arbitrary units and corrected for GAPDH density were 0.1056 ± 0.021 for asthmatic subjects (n = 7) and 0.040 ± 0.008 for controls (n = 5). The difference was significant (P < 0.05).

To determine how specific the smMLCK-108 message changes are to the asthmatic state, we carried out measurements of the message in endobronchial biopsies obtained from subjects with allergic rhinitis but who had no evidence of airway hyperreactivity as tested by bronchoprovocation tests (n = 6). Data are not shown. No difference in densitometric density of these bands was seen when compared with those of nonallergic controls; allergic rhinitics: 0.01 ± 0.03 arbitrary units, nonallergic controls: 0.014 ± 0.004. The data that included those from the three groups, asthmatics, nonallergic controls, and allergic (rhinitic) controls, were analyzed using ANOVA and Duncan’s new mul-

tiple-range test described in MATERIALS AND METHODS. Mean data from the asthmatics were significantly different from the other groups. We concluded that the increased kinase message content seen in asthmatic BSMC is specific.
We reported that airway smooth muscle cells. We also measured the mRNA content for myosin heavy chain (MHC) and because this cannot be measured in single cells, we measured the mRNA content for SM-A and SM-B, again employing RT-PCR. Only bands for the message for SM-A were seen (Fig. 3), which indicates that SM-B is not present in either control or asthmatic cells. These samples were from the same patients whose smMLCK mRNA data are shown in Fig. 2. The numbering of the lanes is also the same. Laser densitometry showed no significant difference in abundance of message between control and asthmatic subjects. Because the bands for myosin heavy chain were of equal density and width in all lanes, they were used to normalize the loadings in the different lanes for smMLCK mRNA. Similar results were obtained for GAPDH loading. The observation that smMLCK-108 mRNA was increased relative to GAPDH and to smMHC contents proves there was a true increase even after normalization.

**DISCUSSION**

Changes in contractile properties of the asthmatic bronchial smooth muscle cells. We reported that airway smooth muscle strips from sensitized animals demonstrate increased $V_o$ and $\Delta L_{max}$ (11, 24). We have now reported similar alterations in intrinsic contractile properties of single BSMC obtained from airways of asthmatic subjects. Moreover, the RT-PCR analysis of asthmatic BSMC showed increased expression of smMLCK-108 mRNA suggesting a molecular basis for the observed change in contractility.

The amount and properties of extracellular connective tissue in smooth muscle strip could affect their mechanical properties (4, 18). Thus results obtained from experiments conducted at tissue level cannot be extrapolated to explain behavior at cellular level for which, however, studies of individual single cells are necessary. The mean data presented here were obtained from 10–20 (for every given subject) cells with a relatively low variability. On the basis of this we believe that the direct measurement of contractile properties at single cell level provides direct evidence to support the hypothesis that airway smooth muscle contractility is increased in asthma; this is an important component of asthma pathogenesis. The cell sampling per subject we believe was adequate, since the 10–20 cells represent 5–10% of all the cells present in the bronchial smooth muscle, which is a very thin layer.

In this study, the RT-PCR analysis was undertaken for two reasons: the first, that it is not possible to measure individual proteins in a small cell sample, the second, that mRNA is a more sensitive measure of change than the protein expression. We made the assumption that the message was an indicator of protein product content. Meer and Eddinger (19) showed that this was so in vascular smooth muscle. We also had to make the further assumption that the stability of the mRNA and smMLCK-108 was unchanged between the two studied groups of subjects.

RNA was extracted from a pool of 6–8 bronchial biopsies per subject, and it is likely that there were some variations in smooth muscle contents between biopsies and subjects. Because we found no obvious difference in SM-A and SM-B mRNA contents between subjects, the variation in BSMC content in the pool of bronchial biopsies was likely not important, at least not enough to explain the difference observed in smMLCK mRNA content. The mechanical and biochemical studies were conducted on biopsies from two subpopulations randomly selected from a fairly well-defined population of mild to moderately severe asthmatics. The paucity of cells obtained from a given subject necessitated the two samples. The two groups were matched as closely as possible, and comparison of the relevant data shows no significant difference between them. This enabled us to conclude that the
samples were indeed drawn from the one homogeneous population.

Measurement of \( V_o \). The exact measurement of \( V_o \) in smooth muscle has not been easy. In skeletal muscle where single crossbridge mechanics can be measured, the T2 transient reported by Huxley and Niedergeke (9) provides records for valid \( V_o \) measurement. In strips of striated and smooth muscle, the problem is that the initial part of the shortening is artefactual because of the presence of lever overshoot. At later points in time, the velocity is artefactually slow as the internal resistor of the cell becomes compressed and loads the contractile element internally, leading to a spuriously low \( V_o \). Stephens et al. (23) published data describing the compliance of the internal resistor for smooth muscle. The results indicate that by 300–500 ms the resistor would load the contractile element significantly. No real solution to the problem of how to measure \( V_o \) exactly exists, and currently we measure \( V_o \) at 100 ms after onset of shortening in human and canine BSMC.

Role of increased shortening velocity \( (V_o) \) in the production of bronchospasm and the relationship between \( V_o \) and \( \Delta L_{max} \). In RESULTS we presented data indicating \( V_o \) was significantly increased in asthmatic BSMC. In contracting canine smooth muscle strips whose contraction time is 10 s (24), at 37°C, >75% of the measured shortening is completed within the first 1.5 s. Similar results are observed with human BSMC, where 50% of the shortening at 22°C (Fig. 1) and 75% at 37°C are complete within 4 s.

During the course of shortening, initial velocities are high and are functions of the activity of normally cycling crossbridges whose MLC20 is phosphorylated at serine-19 (11). This phase is succeeded by a phase of progressively diminishing velocities due to the operation of slowly cycling cross bridges, which are four times slower than the faster cycling bridges active in the first 1.5 s of shortening (11). The former are created through dephosphorylation of MLC20 by a specific myosin light chain phosphatase denoted by PP1M (20, 25). Therefore, \( \Delta L_{max} \) and \( V_o \) are closely interdependent. Similarly, in cardiac muscle, velocity of shortening is a critical determinant of stroke output, because muscle activation duration is limited due to the twitch characteristics of the muscle. The importance of the early phase of shortening in airway smooth muscle contraction has not been adequately recognized to date. Moreover, analyzing shortening data at steady state, i.e., at the plateau of shortening (10 s) may be misleading. Although it will show that the sensitized muscle shortens more than control in toto, it will not identify the fact that most of the shortening occurred in the first 1.5 s, at which time the regulating enzyme (smMLCK-108) is quite different from that at 10 s (PP1M). It is for this reason that we studied smMLCK-108 and not PP1M.

The current study demonstrates that asthmatic BSMC shortened more than normal cells. The one-third increase of \( \Delta L_{max} \) we observed is pathophysiologically significant. If this occurred in vivo, it would result in 81-fold increase in airway resistance as predicted by Poiseuille’s law and would account for the dyspnea that occurs during an asthma attack. It must be admitted that the length change detected represents an upper bound in view of the fact that the cells shortened against zero load. That involvement of smooth muscle contraction is an outstanding feature of both the acute and acute-on-chronic asthma attack is supported by the fact that \( \beta_2 \)-adrenergic agonists can almost always rapidly relieve the respiratory distress (26).

Molecular mechanism underlying increased contractility of the BSMC. The molecular mechanisms of increased contractility of asthma BSMC are currently unknown. The major difficulty, as pointed out before, stemmed from the limitation in obtaining sufficient tissue from subjects for biochemical studies. Previous studies from sensitized canine airway smooth muscle strips indicated that increased expression of protein content of smMLCK-108 accounted for the increased contractile properties of the muscle (11), which are mainly regulated by this enzyme. It must be remembered that unlike striated muscle, smooth muscle myosin ATPase is a very weak ATPase and is stimulated by 10.220.32.247 on August 27, 2017 http://ajplung.physiology.org/ Downloaded from by others in passively sensitized human airway smooth muscles (3). Our RT-PCR studies conducted on isolated cells suggest that the mechanism responsible for the increased contractility of single asthmatic airway smooth muscle is associated with increased abundance of the smMLCK-108 message. If one assumes that the content of the expressed protein is a function of the abundance of message abundance, our findings would explain the increased contractility of the asthmatic airway smooth muscle cell. Furthermore, support for this conclusion is obtained from our canine experiments (11), in which bronchial and tracheal smooth muscle strips from ragweed pollen-sensitized animals showed the same qualitative and quantitative increases in maximum velocity and capacity of shortening as shown here by the single cells obtain from bronchi of human asthmatics. The canine tissues showed increases in total MLCK content. In these experiments the protein was measured directly by Western blots and specific antibodies. Thus the human MLCK mRNA and the canine MLCK protein contents showed the same directional changes. The fact that specific activity of the kinase is unchanged with sensitization (11) indicates that the molecule and its intrinsic activity are unchanged. However, the increased total content would produce phosphorylation of increased amounts of the MLC20 and increased actin-activated, i.e., myosin Mg\(^{2+}\), ATPase activity. Another possible reason for the increased content is a change in
the stability of the protein. Turnover studies to evaluate this possibility are planned for the future in strips of bronchial smooth muscle since the study is not feasible in single cells.

With respect to the validity of the RT-PCR measurements, it must be acknowledged that sequencing studies of the product were not carried out. However, since we detected only one band we surmised that nonspecific messages were not copied. Furthermore the number of base pairs in the band matched those expected for smMLCK message.

Another question relates to the contribution of non-muscle MLCK of molecular mass 210 (nmMLCK-210) to the overall phosphorylation. We have reported (16) that in canine sensitized smooth muscle, the content of nmMLCK-210 is <15% of total MLCK and that the phosphorylation it produces is >30% of the maximal phosphorylation produced. We conclude that the role of nmMLCK-210 in sensitized airway smooth muscle is minor. Differences in amount and distribution of smooth muscle myosin heavy chain isoenzymes would also affect smooth muscle contractile properties. In sensitized canine airway smooth muscle, no increase in SM-A expression was found, and SM-B was absent. We found no increase in expression of SM-A in human asthmatic airway smooth muscle and no expression of SM-B. Kelley et al. (12) have reported that SM-B is present only in phasic smooth muscle. They and others have shown that only SM-A is present in tonic smooth muscle (21). Consequently, the absence of SM-B expression in human airway smooth muscle was not unexpected, since this muscle is of tonic type. Proteins such as tropomyosin, calponin, caldesmon, and the 17-kDa essential myosin light chain are also important in regulation of smooth muscle contraction. Their roles in sensitized bronchial smooth muscle need further investigation.

In conclusion, the endobronchial biopsy technique enables adequate numbers of subjects to be sampled to allow conclusions to be made with statistical significance. The present study demonstrates that human asthmatic BSMC possess significantly increased contractile properties compared with control BSMC. Moreover it suggests that one likely cause for this increase is the presence of an increased BSMC content of smMLCK, which brings about increased actomyosin ATPase activity and bronchospasm.

The smMLCK protein content could not be measured in the 10–20 cells obtained from each subject because of technical difficulty. However, the RT-PCR measurements that were performed showed that the message was significantly increased, and assuming that its stability is not changed and that the message could be used as an indicator of protein content, we concluded that the biochemical changes accounted for the increased contractility of BSMC obtained from asthmatic subjects. Further support for this conclusion is obtained from our canine experiments, in which bronchial and tracheal smooth muscle strips from ragweed pollen-sensitized animals showed almost exactly the same qualitative and quantitative increases in $V_o$ and $L_{max}$ as were shown by the single cells obtained from human asthmatics. The canine tissues showed increases in total smMLCK content. In these experiments the protein was measured directly by Western blots using specific antibodies. Thus the human smMLCK mRNA and the canine smMLCK protein contents showed the same directional changes.

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