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The relevance of resting tension to responsiveness and inherent tone of human bronchial smooth muscle

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1 In the present study the effects of resting tension on isometric responses of human airway smooth muscle to contractile and relaxant stimuli were investigated. Also, its effects on inherent smooth muscle tone were examined, in an effort to determine a pre-defined resting tension which can be considered optimal for *in vitro* studies.

2 Bronchial ring preparations (2–4 mm internal diameter) were suspended in tissue baths at a range of levels of resting tension (200–1600 mg). The responses to electrical field stimulation (EFS, 30 V, 0.2 ms, 8–30 Hz), carbachol (3 μM), isoprenaline (1 μM) and the 5-lipoxygenase inhibitor, zileuton (10 μM) were investigated along with the ability of the preparations to recover stable resting tension after 60 min of washing post challenge.

3 EFS induced monophasic contractions at low stimulation frequency (8 Hz). However, with increasing frequency and tension (15 and 30 Hz; > 400 mg) contractile responses were accompanied by a relaxant component. The magnitude of contractile responses increased with increasing resting tension to a plateau at between 500 and 700 mg. Relaxant responses when present, increased in magnitude with increasing resting tension.

4 The level of resting tension did not significantly alter responses to carbachol, but tissues at tensions $\geqslant$ 1000 mg showed poor tension recovery after washing. Isoprenaline-induced relaxations increased with increasing resting tension ($P<0.05$) and tension recovery after washing was complete. The 5-lipoxygenase sensitive portion of the tension ($33\pm4\%$) was not altered by the level of resting tension.

5 These results suggest that in human bronchial ring preparations of 2–4 mm internal diameter, resting tensions within the range 400–1000 mg could be considered optimal for isometric tension recordings of protocols involving both contraction and relaxation procedures.

Keywords: Human bronchial smooth muscle; carbachol; isoprenaline; 5-lipoxygenase inhibition; electrical field stimulation

Introduction

The tension applied to a smooth muscle preparation at the start of an isometric *in vitro* experiment (resting tension) is an important factor in determining subsequent responsiveness since this tension determines (a) tissue length and (b) development of active force which increases with length until an optimum L_{max}, is achieved (Stephens & Van Niekerk, 1977; Hulsmann & De Jongste, 1993). Studies with preparations of human bronchial smooth muscle have been presented in the literature since the early 1980s, with a variety of experimental conditions (Finney et al., 1985; Stretton *et al*., 1990; Molimard *et al*., 1994; Ward *et al*., 1993). However, perhaps the most notable difference is the large range of resting tensions used (0.5–2.5 g). The limited availability of human tissue has meant that rigorous testing of the optimal conditions for isometric experiments in these preparations has often not been possible. Ideally these conditions should be evaluated for each individual tissue preparation, but this is often not practical. Therefore, in the present study the importance of resting tension in determining the responsiveness of tissues to contractile and relaxant agents, was investigated, as well as electrical field stimulation, and the influence of resting tension on inherent tone in human airways (Rabe *et al*., 1993; Ellis & Undem, 1994).

These studies were performed in an effort to determine a resting tension, or range of resting tensions, in human bronchial ring preparations of a given size (2–4 mm internal diameter), which could be considered optimal. Since a number of experimental protocols involve (a) determination of maximal contractile and/or relaxant responses, for standardizing electrical field stimulation responses (Ward et al., 1993) or (b) repeated concentration-effect curves with stable baseline tensions between curves (Watson *et al*., 1995), these criteria were used as indicies of having achieved optimal conditons. Over a range of resting tensions (200–1600 mg), the magnitude of the contractile response to carbachol or relaxant response to isoprenaline, and the ability of the tissues to re-establish tension after washing and re-equilibrating, were studied. The magnitude and nature of isometric responses to electrical field stimulation were also studied over a range of tensions (100–1000 mg), to determine whether optimal conditions for observing responses to exogenous agents were equivalent to such agents released endogenously through nerve stimulation.

It has been shown that inherent tone in human airways is primarily the result of 5-lipoxygenase metabolites (Ellis & Undem, 1994), but it is unclear what stimulates the production of these metabolites. Since it is conceivable that stretching of the tissue may result in increase production of 5-lipoxygenase metabolites, additional experiments were performed to investigate the influence of increasing resting tension on inherent tone and the relative contribution of 5-lipoxygenase metabolites. This was achieved by studying the effects of the 5-lipoxygenase inhibitor, zileuton, at different levels of resting tension.

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A preliminary account of this work has been presented to the American Thoracic Society (Watson *et al.*, 1996).

Methods

Small bronchi with an internal diameter of 2–4 mm were dissected from macroscopically normal bronchial tissue obtained from 29 patients undergoing surgery for lung cancer (20 male, 9 female; mean age 60 range: 21–86 years). Three patients were taking theophylline, steroids and anticholinergic agents, one patient was taking theophylline alone and two were taking anticholinergic agents and β-adrenoceptor agonists via a metered dose inhaler, as required. Therapy was withdrawn 24 h before surgery and no apparent difference in responsiveness was observed in tissue from the six patients receiving therapy. Tissues were placed in oxygenated (95% O_2: 5% CO_2) modified Krebs buffer (composition (mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 0.6, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1) at 4°C and were used within 48 h. Ring segments, 3–4 mm in length (a total of 159), were mounted in 10 ml organ baths containing oxygenated modified Krebs buffer (pH 7.4, 37°C) and equilibrated for at least 60 min before the experimental protocols were begun. Tissues were suspended between wire supports; one held fast at the bottom of the organ bath and the other attached to a transducer, which was fixed to a rack and pinion to enable fine adjustment of resting tension.

Responses to electrical field stimulation (EFS)

In order to determine the effect of varying levels of resting tension on EFS-induced responses, each level of tension was studied in a single tissue, since it was determined previously that the magnitude and nature of EFS-induced responses in human bronchial tissue vary greatly, not only between tissues from different individuals, but also between adjacent tissues from the same individual (see Coleman *et al.*, 1996).

Tissues were stimulated serially with the aid of a computerized timing system by use of a Harvard stimulator which delivered square wave pulses of 0.2 ms, 30 V at either 8, 15 or 30 Hz for 10 s every 120 s. After equilibrating at 100 mg tension, the stimulator was turned off for 120 s and the tension was increased by 100 mg by raising the transducer via the rack and pinion. The stimulator was then turned on for four stimulations (8 min) before the process was repeated, until a final tension of 1000 mg was achieved (see Figure 1).

Responses to carbachol

Eight ring segments, prepared from tissue from the same individual, were equilibrated for at least 60 min at one of the following resting tensions; 200, 400, 600, 800, 1000, 1200, 1400, or 1600 mg. Once a stable tension was achieved, 3 μM carbachol was applied. When the maximum contraction was obtained, tissues were washed and re-equilibrated for a further 60 min and the new level of tension to which the tissues returned was recorded (recovery tension). At the end of the experiments the mg wet weight of each tissue was determined. This experimental protocol was performed six times in tissues from 6 different individuals.

Responses to isoprenaline

In another eight ring segments, prepared from tissue from the same individual, the protocol outlined above was repeated with the exception that 1μM isoprenaline was added in place of the carbachol. At the end of the experiments the mg wet weight of each tissue was determined.

Responses to zileuton

The protocol outlined above was again repeated, with the exception that 10 μM zileuton was added in place of carbachol and tissues were equilibrated for 60 min. After this time tissues were relaxed with 1 μM isoprenaline. Isoprenaline was then washed from the bath and tissues were re-equilibrated for a further 60 min in the continued presence of zileuton (10 μM), to determine the level of tension to which tissues recovered in the presence of 5-lipoxygenase inhibition. At the end of the experiments the mg wet weight of each tissue was determined.

Data analysis

All responses were recorded as changes in isometric tension (mg). Statistical analysis of the data was performed by use of

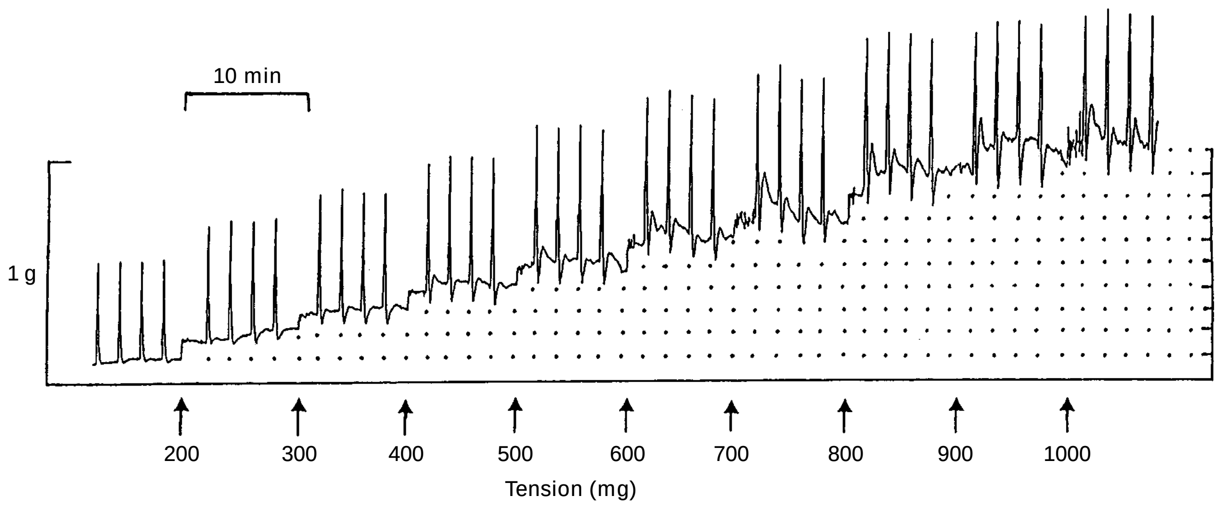

Figure 1 Polygraph trace outlining the protocol employed and showing the effect of increasing tension on the response to electrical field stimulation (30 V, 30 Hz, 0.2 ms for 10 s every 120 s) in a human bronchial smooth muscle ring preparation.

paired and unpaired Student's *t* test where appropriate. Repeated measures analysis of variance was performed to determine if differences between responses at the various tension levels were significant. When significance was found, a Student-Newman-Keuls multiple comparisons test was performed to identify the different groups. Where appropriate, a test for the significance of a linear trend was also performed. $P<0.05$ was considered significant and all values quoted are the mean ± s.e.mean of at least 5 experiments with tissues derived from different individuals.

Materials

Carbachol, isoprenaline, atropine, propranolol, tetrodotoxin and Nω-nitro-L-arginine methyl ester were obtained from Sigma Chemical Company (Deisenhofen, Germany). Zileuton (N-(1-(benzo-[b]-thien-2-yl)ethyl)-N-hydroxyurea) (Abbott-64077) was a generous gift from Abbott Laboratories (Abbott Park, Illinois, U.S.A.). All drug solutions were prepared in distilled water, with the exception of zileuton which was prepared as a 10 mM stock solution in equal volumes of 0.1 M NaOH and distilled water. Dilutions of stock solutions were made with distilled water.

Results

Responses to electrical field stimulation

Electrical field stimulation consistently produced a contraction which was sustained for the duration of the stimulation period (10 s). At low stimulation frequency (8 Hz) the contractile response was monophasic in all tissues at all tensions (Figure 2). Monophasic contractions were obtained in all tissues stimulated at 15 Hz, when the tension was below 400 mg. However, at 400 mg tension and greater three of the 5 preparations exhibited a biphasic response; contraction followed by a relaxation (Figure 2). At 30 Hz, the contractile response was smaller in magnitude than at 15 Hz, but was consistently followed by a relaxant response in all tissues. The magnitude of both the contractile and the relaxant components increased with increasing tension, showing a significant linear trend in both cases. However, the magnitude of these responses had reached a plateau and had begun to fall within the range of tensions studied (Figures 1 and 2b).

Stimulation-induced contractions were abolished by atropine (0.1 μM) and tetrodotoxin (0.1 μM) confirming the cholinergic neuronal nature of these responses. Relaxant responses were unaltered by propranolol (1 μM), but were abolished by Nω-nitro-L-arginine methyl ester (0.1 mM, L-NAME), confirming the non-adrenergic non-cholinergic (i-NANC) nature of these inhibitory responses (Figure 3).

Responses to carbachol

Carbachol caused contractions in all tissues, which were not significantly different between tissues at the different levels of resting tension when expressed either as absolute changes in mg tension or as a ratio of tissue wet weights (Figure 4a and b).

Tissues at resting tensions between 200 and 800 mg recovered, after the 60 min re-equilibration period, to a level of tension which was not significantly different from that recorded immediately before addition of carbachol. However, at resting tensions of 1000 mg and greater, tissues recovered to levels of tension which were significantly lower (Figure 4c). This fall in recovery tension with increasing levels of resting tension showed a significant linear trend.

Responses to isoprenaline

Isoprenaline caused relaxation in all tissues. The magnitude of relaxations increased significantly with the level of resting tension, whether expressed as absolute changes in mg tension or a ratio of tissue wet weight (Figure 5a and b). This increase with increasing resting tension showed a significant linear trend but at the higher levels of tension the response appeared to have reached a plateau. Expressing data as a percentage of the resting tension, relaxations declined significantly with

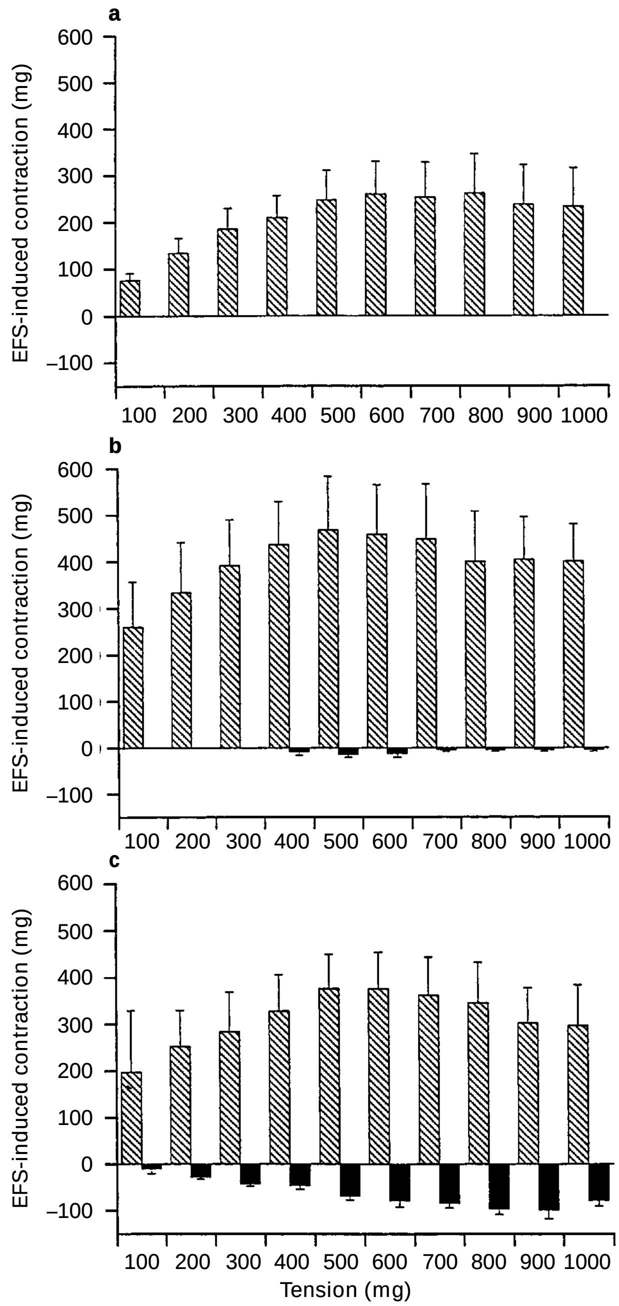

Figure 2 EFS-induced responses of human bronchial smooth muscle with increasing levels of tension at stimulation frequencies of (a) 8 Hz, (b) 15 Hz and (c) 30 Hz. Shaded columns represent the contractile component, while solid columns represent the relaxant component associated with responses at each level of tension. Data are the mean ± s.e. of at least 5 experiments at each frequency, with tissue derived from different individuals.

Figure 3 Polygraph trace of electrical field stimulation (EFS: 30 V, 30 Hz, 0.2 ms for 10 s every 120 s) -induced responses of a human bronchial ring preparation, demonstrating the cholinergic nature of contractile responses and non-adrenergic non-cholinergic (NANC) nature of inhibitory responses as indicated by their respective sensitivities to atropine (0.1 μM) and L-NAME (0.1 mM), respectively.

increasing levels of tension (Figure 5c) showing an opposite linear trend. However, in the range 800–1400 mg, responses were not significantly different from each other when expressed either as mg tension changes or as % of resting tension.

After the 60 min re-equilibration period, all tissues recovered to a level which was not significantly different from that recorded immediately before addition of isoprenaline, with the exception of those at 1600 mg, which recovered to a level which was significantly lower (137 ± 17 mg fall in tension).

Responses to zileuton

Zileuton caused a fall in tension over the 60 min exposure period. The magnitude of the fall, like that induced by isoprenaline, increased with increasing resting tension (Figure 6a and b) showing a significant linear trend. However, when the zileuton-induced fall in tension was expressed as a percentage of the resting tension, there was no significant difference between preparations (33 ± 4% of the resting response, Figure 6c). Addition of isoprenaline to zileuton-treated tissues caused a further fall in tension in all but two preparations, indicating residual tone after 5-lipoxygenase inhibition. After the isoprenaline was removed by washing, tissues recovered to a level of tension which was not significantly different from that before isoprenaline, confirming the presence of inherent tone in addition to the 5-lipoxygenase-sensitive component.

Discussion

In the present study we investigated the relevance of different levels of resting tension in determining isometric contractile and relaxant responses in human bronchial smooth muscle *in vitro* and the relative contribution of 5-lipoxygenase products to inherent tone. The purpose of this study was to determine whether a resting tension, or range of resting tensions, could be determined within which human bronchial ring preparations of a given size (2–4 mm internal diameter) could be considered to be at optimal tension. In addition, the relative contribution of 5-lipoxygenase products to tone at the different levels of resting tension has been assessed.

Low pulse width electrical field stimulation of human bronchial smooth muscle, such as was used in the present study, stimulates cholinergic and non-adrenergic non-cholinergic inhibitory (i-NANC) nerves embedded within the airway walls (Laitinen, 1985; Barnes, 1989; Barnes *et al*., 1991). The neuronal, cholinergic and i-NANC nature of these responses was confirmed in the present study, by the inhibition of EFS-induced responses by tetrodotoxin, atropine and L-NAME, respectively. The magnitude and nature of EFS-induced responses varies greatly between preparations, which is consistent with previous observations (Coleman *et al*., 1996) and for this reason the effects of different levels of tension were investigated within the same preparations.

Cholinergic contractile responses were consistently accompanied by i-NANC relaxations in tissues stimulated at 30 Hz, regardless of the level of resting tension. However, at 15 Hz stimulation, relaxation was only observed in tissues once the level of resting tension was raised to 400 mg and above. No relaxation was observed in tissues stimulated at 8 Hz regardless of the level of tension. Therefore, contractile responses increased with resting tension to a plateau at between 500 and 700 mg and at higher stimulation frequencies the level of resting tension determined whether or not a relaxant component could be detected. The resting tension selected as optimal would therefore depend upon the experimental protocol to be used; the cholinergic component of the response being maximal between 500 and 700 mg tension, while the i-NANC response was maximal between 800 and 900 mg tension in the present study.

The magnitude of the contractile response to carbachol was independent of the level of resting tension, in the range investigated in the present study (200–1600 mg). This suggests that any level of tension within this range could be considered optimal for observing contractile responses to this agonist and this appeared to be true of the data, whether expressed as absolute changes in mg tension or as a ratio of the mg wet weight of the tissues. However, tissues which were contracted from 1000 mg and higher did not recover completely after a 60 min period of repeated washing. In these tissues a new level of tension was established which was significantly lower than that before addition of carbachol. By contrast, the magnitude of relaxant responses to isoprenaline was dependent on the level of resting tension, but tissues recovered to their original tension after washing (only tissues at 1600 mg showing a significant reduction). The inhibitory effects of zileuton on tone, like those of isoprenaline, were also dependent on the level of resting tension. However, when expressed as a percentage of this tension, the relative fall with zileuton was not significantly altered by the level of tension. The zileuton-sensitive component of the tone (33 ± 4%) is in keeping with the value of 33 ± 6% obtained by Ellis & Undem (1994). The demonstration of residual tone in the presence of 5-lipoxygenase inhibition is also consistent with previous findings and it was suggested that histamine is responsible for the remaining component (Ellis & Undem, 1994). It is unlikely that the residual tone in the presence of zileuton (10 μM) reflects incomplete inhibition of the 5-lipoxygenase, since the IC_{50} concentrations for this compound at inhibiting 5-lipoxygenase are in the range 0.3 – 0.5 μM (Carter *et al*., 1991).

It is also apparent from these results that the stability of the preparation, as reflected by its ability to undergo maximal

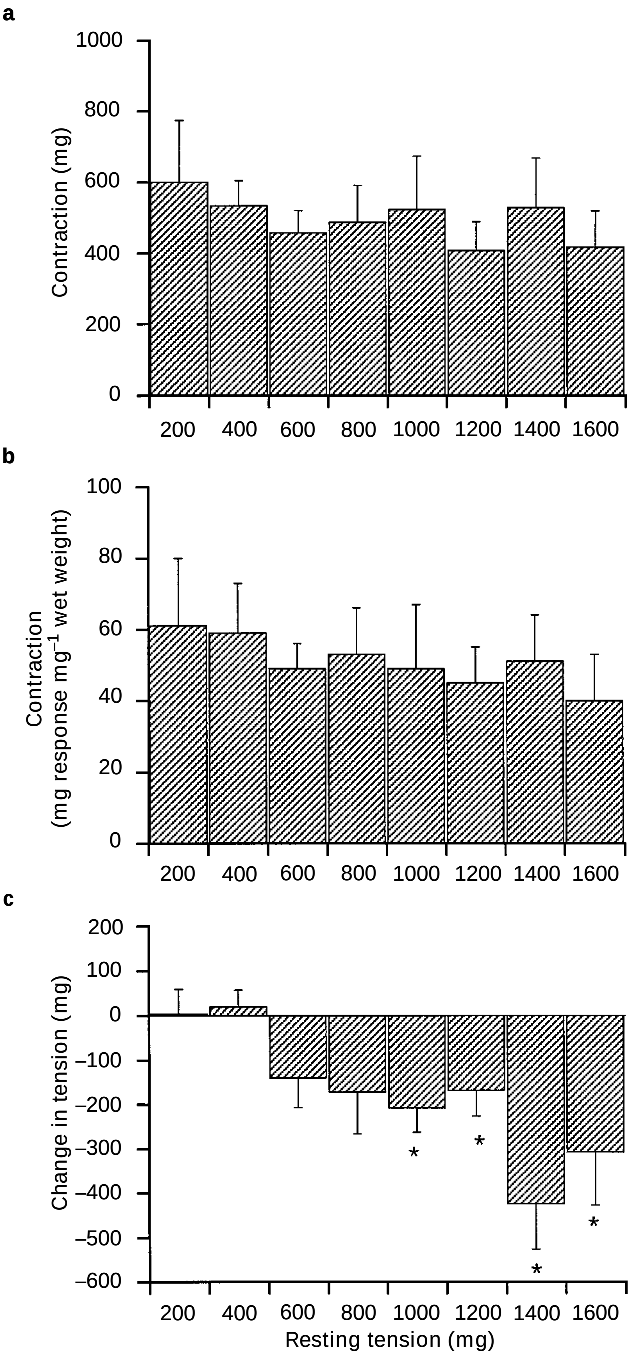

Figure 4 Increase in tension induced by carbachol (3 μM) in human bronchial smooth muscle equilibrated at a range of resting tensions. Data are expressed as (a) absolute tension changes in mg and (b) as a ratio of the mg wet weight of each individual tissue. (c) The tension change associated with challenge which is the change in tension 60 min after washing out the effects of carbachol at the various levels of resting tension (i.e. recovery tension – tension level before carbachol). Data are the mean ± s.e. of 6 experiments for each level of tension, with tissues derived from 6 different individuals. $*P<0.02$, by paired Student's *t* test to compare level before and after carbachol.

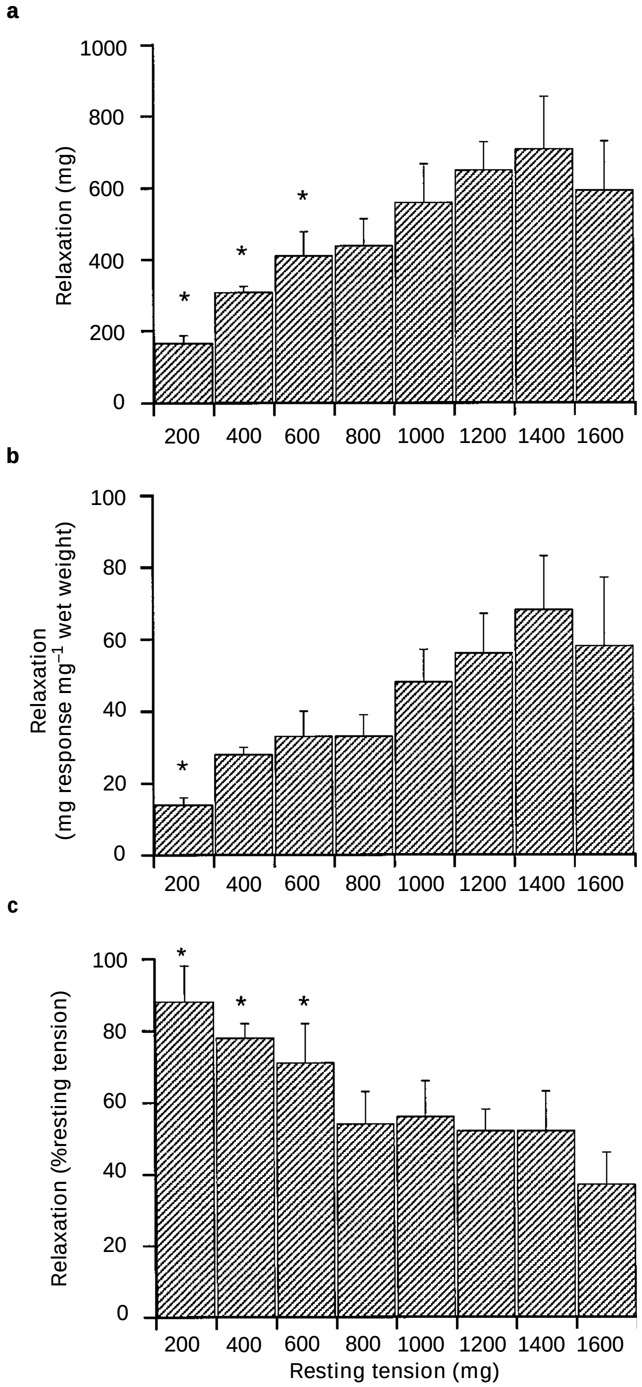

Figure 5 Fall in tension induced by isoprenaline (1 μM) in human bronchial smooth muscle equilibrated at a range of resting tensions. Data are expressed as (a) absolute mg tension changes, (b) as a ratio of the mg wet weight of each individual tissue and (c) as a percentage of the resting tension in each individual tissue. Data are the mean ± s.e. of 6 experiments for each level of tension, with tissues derived from 6 different individuals. $*P<0.05$ by Student-Newman-Keuls multiple comparison test for absolute mg tension changes 200 vs 1000 – 1600 mg and 400 vs 1400 mg, for mg tension changes as a ratio of the mg wet weight 200 vs 1200 – 1600 mg and for changes as a percentage of the resting tension 200 vs 1000 – 1600 mg, 400 vs 1600 mg and 600 vs 1600 mg.

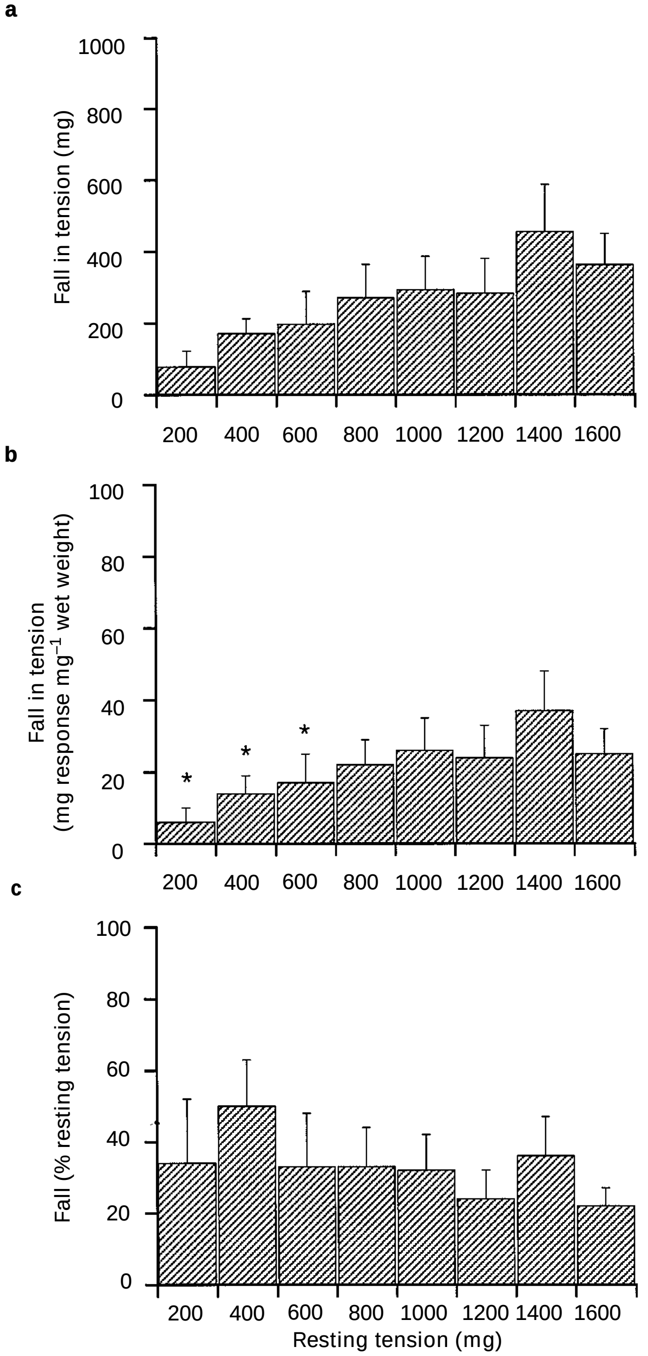

Figure 6 Fall in tension induced by zileuton (10 μM) in human bronchial smooth muscle equilibrated at a range of resting tensions. Data are expressed (a) as absolute mg tension changes, (b) as a ratio of the mg wet weight of each individual tissue and (c) as a percentage of the resting tension in each individual tissue. Data are the mean $\pm$ s.e. of 6 experiments for each level of tension, with tissues derived from 6 different individuals. $*P<0.05$ by Student-Newman-Keuls multiple comparison test. For absolute mg tension changes all comparisons were significantly different with the exception 200 vs 400 mg, 600 vs 800 mg, 800 vs 1000 mg and 1200 vs 1400 mg, for mg, tension changes as a ratio of the mg wet weight 200 vs 800–1600 mg, 400 and 600 vs 1400 mg.

contraction and yet recover to its original level of tension, decreases at levels of resting tension in excess of 1000 mg. Whether this reflects a permanent change in the ability of the muscle to maintain tension is unclear, but the 200 to 400 mg fall in baseline tension which occurs may influence the reproducibility of relaxant responses, which appear to be more dependent on the level of tension. This could become a confounding factor for the analysis of data.

The results of the present study reveal a number of novel findings. Firstly, within the tension range investigated in the present study (200–1600 mg), responses to contractile agonists were tension-independent, while responses to relaxant agonists were tension-dependent. The latter finding applied to responses resulting from either exogenously applied agonists or from the release of such agonists by nerve stimulation. Secondly, the relative contribution of 5-lipoxygenase products to inherent tone in human airways was independent of the level of resting tension of the preparation. Finally, while stimulation parameters were important in determining the nature of EFS-induced responses in human bronchial smooth muscle (monophasic versus biphasic), the level of resting tension influenced the magnitude of i-NANC responses.

The practical significance of these findings is that not all resting tensions are optimal for observing both contractile and relaxant responses and that the choice of experimental protocol will influence the choice of resting tension. However, the present data suggest that in human bronchial ring preparations, with an internal diameter of 2–4 mm, resting tensions within the range 400–1000 mg appear (i) to be optimal for contractile and relaxant responses produced either by exogenous or endogenous agents (EFS) and (ii) to generate a stable inherent tone of which approximately 33% can be attributed to 5-lipoxygenase products.

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