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Involvement of the neurokinin-2 receptor in airway smooth muscle stretch-activated contractions assessed in perfused intact bovine bronchial segments

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Role of NK_2-receptors in ASM stretch-activated contraction

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Abbreviations:

5-HT – 5-hydroxytryptamine (3-(2-aminoethyl)-1*H*-indol-5-ol)

ASM – Airway smooth muscle

CCh – Carbachol (2-carbamoyloxyethyl-trimethyl-azanium)

DI – Deep inspiration

ECM – Extracellular matrix

FRC – Functional residual capacity

L-732,138 – N-Acetyl-L-tryptophan-3,5-bis(trifluoromethyl) benzylester

MEN 10376 – [Tyr^{1},D-Trp^{6,8,9,}Lys^{10}]-NKA(4-10)

NK – Neurokinin

PAR-2 – Protease-activated receptor-2

RAR – Rapidly adapting pulmonary stretch receptors

$R_{stretch,x}$ – contraction evoked by an instantaneous stretch to x cmH_20

SAR – Slowly adapting pulmonary stretch receptors

SP – Substance P

SR48968 – N-[(2S)-4-(4-acetamido-4-phenylpiperidin-1-yl)- 2-(3,4-dichlorophenyl)butyl]-N-methylbenzamide

TLC – Total lung capacity

TRPV1 – Transient receptor potential vanilloid 1

TTX – Tetrodotoxin

VSM – Vascular smooth muscle

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Abstract

The airway response to deep inspirations (DIs) in asthmatics has been shown to be ineffective in producing bronchodilation, and can even cause bronchoconstriction. However, the manner by which a DI is able to cause bronchoconstriction remains ambiguous. We sought to investigate the pathway involved in this stretch-activated contraction, as well as whether this contraction is intrinsic to airway smooth muscle (ASM). Briefly, intact bovine bronchial segments were dissected and side branches ligated, then mounted horizontally in an organ bath. Intraluminal pressure was measured under isovolumic conditions. Instantaneously opening and then closing the tap on a column of fluid 5-30 cm high evoked a sudden increase in intraluminal pressure (equivalent to the height of the column of fluid) followed by a stress relaxation response of the ASM. When tissues were stimulated with carbachol (10^{-8}M) or serotonin (10^{-7}M) for 10 min and the consequent agonist-evoked pressure response was dissipated manually, the response to the same transmural stretch was accompanied by a slowly-developing and prolonged increase in intraluminal pressure. This stretch-activated response was significantly diminished by the stretch-activated cation channel blocker gadolinium (10^{-3}M), the L-type Ca^{2+} channel blockers nifedipine ($2x10^{-6}$M), diltiazem (10^{-5}M), and verapamil (10^{-5}M), the sensory neurotoxin capsaicin (10^{-5}M), and the NK_2-receptor antagonists MEN 10376 (10^{-5}M) and SR48968 ($3x10^{-6}$M). These results show the ability of isolated airways to exhibit stretch-activated contractions, and suggest a role for stretch-activated cation channels, sensory afferent neurons, the neurotransmitter NKA, as well as L-type Ca^{2+} channels in these isolated airway responses.

Introduction

Mechanotransduction, defined as the conversion of mechanical stress into biochemical information, is essential to the proper functioning of cells and organ systems (Hallworth, 1995). In the vasculature, blood pressure is strictly regulated by signaling pathways that respond to mechanical stress to ensure the precise control of blood flow under physiological conditions ranging from vigorous exercise to complete rest (Folkow, 1990). In 1902, Bayliss performed experiments using dog hindlimb which showed blood vessels responding to increased transmural pressure by constricting. This phenomenon was later termed a *myogenic response*, as it was an intrinsic property of the vascular smooth muscle (VSM) independent of neural, metabolic, or hormonal input (Davis and Hill, 1999). Similar to the vasculature, the airways are also constantly subjected to mechanical stress due to the inflation and deflation of the lungs. This stress produces both relaxant and constrictor responses in airway smooth muscle (ASM) (Maksym et al., 2005). Thus, airway stretch is suggested to be either beneficial (bronchodilatory) in healthy individuals or harmful (leading to airway hyperresponsiveness) in asthmatics.

A deep inspiration (DI) is clinically measured as a breath taken from functional residual capacity (FRC) to total lung capacity (TLC). DIs produce bronchodilation in non-asthmatic individuals, whereas in asthmatics they do not convey this protective effect, and can even cause bronchoconstriction (Lim et al., 1987;Salome et al., 2003). The mechanisms by which a DI is able to cause bronchoconstriction remain ambiguous. One suggestion is that smooth muscle activation and tension generation cause an increase in ASM stiffness to the point where it stretches little during a DI. This can subsequently cause the ASM to enter a frozen state, where it stays in a high-stiffness, low-hysteresis latch state (An et al., 2007). Another theory suggests that a DI-induced bronchoconstriction is a peripheral parenchymal hysteresis-associated event,

related to the lung pressure–volume hysteresis curve. Following lung inflation to TLC during a DI, the lower recoil pressures during deflation at any given volume can lead to smaller airways than before the DI was performed, due to unloading of the ASM, which narrows the airways more than it would have otherwise (Lim et al., 1987). Since the failure of bronchodilation following a DI depends on the degree of airway obstruction in asthma, severe asthmatics lose more than they gain when performing a DI. However, the airway inflammation and remodelling present in asthmatic airways may also add to the increased ASM contractility following stretch, by the release of stimuli that can prime the contractile apparatus to react excessively in the presence of stretch. Passive sensitization to IgE has been shown to unmask stretch-activated contractions in human airways in vitro (Mitchell et al., 1997), suggesting a role for inflammatory mediators.

In VSM, stretch-activated contractions are mediated in part though the release of substance P (SP) from sensory neurons (Scotland et al., 2004). ASM tone is regulated in part by a subset of myelinated and unmyelinated sensory nerves, such as slowly-adapting (SAR) and rapidly-adapting (RAR) pulmonary stretch receptors, as well as C-fibre receptors. C-fibres terminate in the airway epithelium and in proximity to the ASM deep within the submucosa. These are nociceptive and respond to many of the mediators released by tissue damage. They are also polymodal and respond to both mechanical and chemical stimuli such as the sensory neurotoxin capsaicin (Widdicombe, 2003). Capsaicin mediates its excitatory effects by binding to the vanilloid receptor, TRPV1 (Gunthorpe et al., 2002;Guo et al., 1999). When activated, C-fibre receptors release sensory neuropeptides including SP and neurokinin-A (NKA), both of which can exert a bronchoconstrictor response (Joos et al., 2000).

Recent single cell and tissue bath studies have shown that ASM *per se* can contract in response to stretch (Maksym et al., 2005;Noble et al., 2004). These responses are mediated by the opening of mechanically-gated stretch-activated cation channels (Hamill and Martinac, 2001). Pretreatment of guinea pig tracheal ASM strips with the stretch-activated cation channel blocker gadolinium (Gd^{3+}) significantly decreased isometric force generation after stretch (Ito et al., 2006).

In this study, we set out to investigate the effect of acute airway stretch on agonist-induced contraction in bovine bronchial segments, as well as the possibility that SP and NKA release can mediate stretch-activated contractions in these tissues. Moreover, we assessed the potential involvement of stretch-activated cation channels using the isolated bronchial segment technique previously described by (Mitchell et al., 1989).

Methods

Animals. All experimental procedures were approved by the McMaster University Animal Care Committee (McMaster University, Hamilton, ON, Canada) and conform to the guidelines set by the Canadian Council on Animal Care (Ottawa, ON, Canada). Lower lobes of lung were obtained from cows (200–500 kg) euthanized at a local abattoir and transported to the laboratory in ice-cold modified Krebs buffer solution (116 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11mM D-glucose, 0.01 mM indomethacin), saturated with 95% oxygen - 5% carbon dioxide to maintain pH at 7.4. Upon receipt of the lobes of lung, intact bovine bronchial segments (2 mm diameter, 20 mm length) were carefully dissected free from surrounding parenchyma, excised, and immediately used or stored in modified Krebs solution at 4^{o}C for up to 24 h.

Bronchial segment preparation. Following the dissection and excision of the bronchial segment, side branches were tightly ligated with surgical silk (4-O) as previously mentioned (Khangure et al., 2004;Mitchell et al., 1998). The ligated bronchial segment was then mounted horizontally in a 30 ml Mayflower organ bath (Hugo Sachs Elektronik, March-Hugstetten, Germany) containing warmed modified Krebs buffer solution (37^{o}C) gassed with carbogen (95% O_2 – 5% CO_2) as previously mentioned by (Mitchell and Sparrow, 1994) with modifications. Briefly, both luminal ends of the airway were mounted on adjustable cannulae that allowed airways of different lengths to be mounted. The airway lumen was filled with warmed modified Krebs solution gassed with carbogen *via* a jacketed-reservoir, the height of which set the baseline transmural pressure (~5 cmH_2O). This baseline pressure was selected to simulate the transmural pressure found in relaxed airways (Noble et al., 2007). The connectors at each end of the airway

possessed 3-way taps, which could be opened to flush the airway with modified Krebs solution or closed to make the airway lumen isovolumic. The intraluminal pressure was recorded with a pressure transducer (Hewlett-Packard Medical Products, MA., USA) attached proximally to the airway. The pressure transducer output was fed through a pressure amplifier (Hewlett-Packard Medical Products, MA., USA) and data was digitally recorded using WinDaq DI-720 recording software (DataQ Instruments, OH., USA). Manual transmural pressure variation was induced by varying the height of perfusate in a column manometer attached distally to the cannulated airway.

The airway segment was mounted at 115% of its resting length (the latter being the length of the segment when dissected free from parenchyma at zero transmural pressure). This co-axial stretch has previously been shown to produce increased contractile responses compared to an airway segment mounted at resting length (Khangure et al., 2004). Subsequently, a pressure test was performed to ensure that there were no leaks in the airway. The segment was then left to equilibrate for ~2 hours, during which the lumen and adventitia were regularly washed with fresh modified Krebs solution. Following tissue equilibration, transmural pressure was set to 5 cmH_2O by manually opening the 3-way tap which communicated with the reservoir of Krebs buffer 5 cm higher than the bronchial segment; equilibration of pressures between the two compartments was essentially instantaneous. With the 3-way tap now closed (isovolumic condition), tissues were treated with 60 mM KCl and the contractile response (isovolumic increase in intraluminal pressure) was recorded in order to test viability. After washing four times, baseline pressure was then reset to ~ 5 cmH_2O by opening/closing the tap.

Tissue Baths. Following the tissue viability test, the airway was allowed 20 min of recovery time under isovolumic conditions. Subsequently, electric-field stimulation (EFS) responses were

evoked at 5 min intervals until a uniform response was established (after approx. 3-4 repetitions) under isovolumic conditions. EFS was delivered by a train of pulses (60 volts, 2 ms pulse duration, and frequency of 20 pulses per second), evoked *via* circular electrodes placed above and below the airway in the organ bath, which were connected to a Grass S48 stimulator (Grass Technologies, RI., USA). The airway was then stretched by opening the 3-way tap which now communicated with a column of fluid 10-30 cm in height, allowing the pressure between the two to equilibrate instantaneously, and then closing the tap (*ii* and *iii* in Fig. 1A and 1B); this increased intraluminal pressure was maintained isovolumically for 3 minutes. Intraluminal pressure was then restored to baseline by opening/closing the tap and allowing equilibration with the reservoir 5 cm higher than the tissue (*iv* in Fig. 1A and 1B). The tissue was allowed 5 min recovery time. To mimic the increased airway tone seen in asthmatic airways, this process was repeated following pretreatment with 10^{-8}M carbachol (CCh) or 10^{-7}M serotonin (5-HT) added to the bath solution to induce submaximal ASM tone under isovolumic conditions (*v* in Fig. 1A and 1B). Ten minutes later, at which point agonist-induced tone had reached a plateau, transmural pressure was reset to ~5 cmH_2O (by opening and closing the 3-way tap; *vi* in Fig. 1A and 1B) before re-assessing airway contractile responses to stretch ($R_{stretch}$) (*vii*, *viii* and *ix* in Fig. 1A and 1B). This protocol enabled the investigation of the effects of mechanical stretch on intraluminal pressure generation in the perfused isolated bronchial segment.

Pharmacological interventions. To investigate the pathway involved in airway stretch-activated contractile responses, tissues were treated with a range of different antagonists following assessment of stretch-activated contractions under control conditions (where tissues were pretreated with CCh (10^{-8}M)). The possible role for stretch-activated cation channels was tested

by pretreating for 30 min with gadolinium (Gd^{3+}; 10^{-3}M), while a role for L-type Ca^{2+} channels was assessed by pretreatment for 30 min with nifedipine ($2x10^{-6}$M), verapamil (10^{-5}M) or diltiazem (10^{-5}M). To assess any potential neurogenic component of airway constriction (Canning and Fischer, 2001;Joos et al., 2000;Widdicombe, 2003), we tested the effect of pretreating with: the Na^{+}-channel blocker tetrodotoxin (TTX) (10^{-6}M; 10 min); the sensory excitatory neurotoxin capsaicin (10^{-5}M; 20 min); the neurokinin-1 (NK_1) receptor antagonist L-732,138 (10^{-5}M (data not shown), 10^{-4}M; 30 min); or the NK_2 receptor antagonists MEN 10376 (10^{-7}M (data not shown), 10^{-6}M (data not shown), 10^{-5}M; 30 min) or SR48968 ($3x10^{-6}$M; 30 min).

Chemicals and solvents. L-732,138 was obtained from Biomol International L.P. (PA, USA). MEN 10376 was obtained from LKT Laboratories Inc. (MN, USA). SR 48968 was kindly donated by Sanofi-Synthelabo Recherche (Montpellier, France). All other pharmacological agents were obtained from Sigma–Aldrich (ON, Canada). The 10 mM stock solutions were prepared in distilled water (CCh, 5-HT, diltiazem, Gd^{3+}), dilute acetic acid (TTX), absolute EtOH (nifedipine, verapamil, L-732,138) or DMSO (MEN 10376, SR48968). Dilutions of these were made in physiological medium; the maximal bath concentration of solvents did not exceed 0.1%, which we have found elsewhere to have little or no effect on mechanical activity.

Statistical Analysis. Stretch-activated contractions ($R_{stretch}$) were quantified as the difference between the minima and the maxima observed in the transmural pressure recordings following a sudden isovolumic stretch (Fig. 1A). All responses were reported as means ± SEM; *n* refers to the number of animals. Statistical analyses comparing multiple groups were done using one-way

ANOVA followed by the Bonferroni's multiple comparison *post-hoc* test; Statistical comparisons between paired groups were made using the Paired t-test; $P < 0.05$ was considered statistically significant.

Results

Airway stretch-activated contractions. In resting tissues at a baseline transmural pressure of 5 cmH_2O, instantaneously opening and closing the tap communicating with a column of Krebs 10-30 cm high led to a sudden increase in transmural pressure (presumably accompanied by a small increase in luminal volume, though this was not measured) followed by a prolonged isovolumic stress relaxation response (*i-iii* in Fig. 1A and 1B). After restoring transmural pressure to baseline (by opening/closing the tap communicating with a 5 cm column of fluid and allowing some fluid to escape) (*iv* in Fig. 1A and 1B), the tissue was challenged with CCh (10^{-8}M) under isovolumic conditions (*v* in Fig. 1A and 1B): there was an increase in airway tone shown by a rise in active transmural pressure. When this cholinergic tone had stabilized, we reset transmural pressure to 5 cmH_2O (by opening/closing the tap and allowing fluid to exit the airway; *vi* in Fig. 1A and 1B) and allowed 5 minutes for the tissue to re-equilibrate under those new isovolumic conditions before re-assessing the response to a sudden pressure pulse (10-30 cmH_2O, as described above; *vii-ix* in Fig. 1A and 1B). In contrast to what was seen in the absence of any underlying cholinergic stimulation (above), the instantaneous spike and transient decrease in transmural pressure (stress relaxation) were now followed by a slowly-developing and prolonged contraction ($R_{stretch}$), the magnitude of which increased with increasing pressure pulse amplitude (Fig. 2B). To determine whether that third component of the mechanical response was a uniquely cholinergic phenomenon, we repeated this experiment using 10^{-7}M 5-HT and obtained the same relationship between test pressure pulse amplitude and magnitude of $R_{stretch}$ (Fig. 2C).

To characterize the mechanisms underlying $R_{stretch}$, all subsequent experiments used a standard test pulse of 30 cmH_2O since the contractile response ($R_{stretch,30}$) was maximal at this

point (Fig. 2B and 2C) and since this mirrors the transmural pressure seen during a deep inspiration to TLC in humans (Allen et al., 2005;Scichilone et al., 2004).

Relationship between agonist concentration and $R_{stretch,30}$. Next, we investigated the dependence of $R_{stretch,30}$ upon the degree of excitation produced by agonist-stimulation. Tissues were stimulated with varying concentrations of CCh or 5-HT for 10 minutes, after which transmural pressure was returned to 5 cmH_2O by allowing fluid to exit the lumen of the airway and 5 minutes given before evaluating the response to a transmural pressure pulse of 30 cmH_2O. Even when tissues were stimulated with CCh or 5-HT at concentrations which evoked little or no contractile response of their own (Fig. 3B and 3D), there was a substantial $R_{stretch,30}$ (Fig. 3A and 3C). The latter increased in magnitude with increasing degrees of excitatory stimulation, reaching a peak at 10^{-8}M CCh and 10^{-7}M 5-HT: these agonist concentrations were sub-maximally effective with respect to evoking a direct bronchoconstrictor response.

Effect of stretch-activated cation channel blockade on $R_{stretch,30}$. To investigate whether stretch-activated cation channels are involved in $R_{stretch,30}$, we used a mechanosensitive cation channel blocker Gd^{3+} (Coirault et al., 1999). Control responses were established upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O prior to antagonist treatment. Gd^{3+} (10^{-3}M) significantly reduced airway $R_{stretch,30}$ compared to control (Fig. 4).

Effect of L-Type Ca^{2+} channel blockade on $R_{stretch,30}$. To investigate whether L-type Ca^{2+} channel blockade would affect $R_{stretch,30}$, we established our control responses upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O before treating the airway segments

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with a variety of L-type Ca^{2+} channel blockers for 20 minutes and then re-evaluating $R_{stretch,30}$. Blockers included the dihydropyridine nifedipine, the phenylalkylamine verapamil, and the benzothiazepine diltiazem (Fleckenstein, 1977; Middleton E Jr, 1984; Triggle and Swamy, 1980). Nifedipine ($2x10^{-6}$M), verapamil (10^{-5}M), and diltiazem (10^{-5}M) all abolished $R_{stretch,30}$ (Fig. 5).

Effect of capsaicin and TTX on $R_{stretch,30}$. To determine whether neurogenic mechanisms contributed to this $R_{stretch,30}$, we treated airway segments with the sensory excitatory neurotoxin capsaicin (10^{-5}M; 20 min) to induce desensitization of sensory afferents by causing depletion of neurotransmitters contained within their nerve terminals. Control responses were established upon pretreatment with 10^{-8}M CCh at a transmural pressure load of 30 cmH_2O, before treating the airway segments with neurotoxin. Treatment with 10^{-5}M capsaicin abolished $R_{stretch,30}$ (Fig. 6), thus, showing a vital role for sensory afferent neurons in this phenomenon. Interestingly, TTX (10^{-6}M) did not significantly affect $R_{stretch,30}$ (although we did find it to be sufficient to abolish EFS-evoked responses; data not shown), suggesting that a TTX-resistant neural component might be involved in mediating these contractions (Fig. 6).

Effect of NK_1- and NK_2-receptor antagonists on $R_{stretch,30}$. Our results above suggest the involvement of a TTX-resistant sensory neural mechanism in $R_{stretch,30}$. Thus, we sought to determine the neurotransmitter implicated in these contractions by blocking either NK_1- or NK_2-receptors to assess the role of SP or NKA respectively. The NK_1 receptor antagonist L-732,138 (10^{-4}M) showed no significant effect on $R_{stretch,30}$, suggesting that airway stretch-activated contractions are not mediated by SP release from sensory neurons. A 10-fold lower concentration of L-732,138 (10^{-5}M) elicited no significant effect on $R_{stretch,30}$ (data not shown). Interestingly,

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blockade of NK_2 receptors by either MEN 10376 (10^{-5}M) or SR48968 ($3x10^{-6}$M) caused a significant reduction in $R_{stretch,30}$ suggesting an essential role for NKA (Fig. 7). Concentrations of 10^{-7}M and 10^{-6}M of MEN 10376 caused a dose-dependent but non-significant decrease in $R_{stretch,30}$ (data not shown).

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Discussion

There have been numerous reports of stretch eliciting a contractile response in ASM; however, most of these studies have used ASM cells or strips (Gunst and Russell, 1982;Maksym et al., 2005;Mitchell et al., 1997). Previous studies have also deemed ASM $R_{stretch}$ as a myogenic event (Stephens et al., 1975;Thulesius and Mustafa, 1994), suggesting an intrinsic property of ASM itself. However, an examination of the pathway involved in airway $R_{stretch}$ has previously not been addressed using perfused intact bronchial segments.

Here we describe the ability of perfused bovine bronchial segments to constrict in response to stretch, but only when pretreated with submaximally-effective, or even sub-threshold, concentrations of a contractile agonist (CCh and 5-HT). In Figure 1B we summarize the changes in pressure, volume and muscle length at different points in our experimental protocol. Due to the incompressibility of the liquid within the airway lumen, airway volume and muscle length remain constant during isovolumic conditions. The increase in pressure and muscle length seen in Figure 1B*ii* is attributed to a change in volume of fluid within the airway. The subsequent isovolumic loss of pressure immediately following a test pulse (Fig. 1B*iii*), on the other hand, is attributed to "stress relaxation" in the passive tissues: this may include processes such as fluidization of the cytoskeleton during the stretch and equilibration of the series and parallel elastic elements, although our experimental approach is not able to resolve these. Moreover, we hypothesize that following agonist pretreatment (*v* in Fig. 1A and 1B), the loss of pressure and decrease in muscle length seen in Figure 1B*vi* are related to the volume of fluid expelled from the airway lumen upon opening/closing of the 3-way tap: clearly, the change in muscle length would be proportional to the concentration of agonist used. As such, the changes in airway volume (and muscle length) upon eliciting an $R_{stretch,30}$ in Figure 1B*vii* would have been less than

that seen in Figure 1B*ii*. At this smaller muscle length, the isovolumic stress relaxation is now followed by substantial force generation (Fig. 1B*ix*), which we attribute in part to re-organization of the contractile apparatus, as well as changes occurring in the contractile signaling pathway due to the presence of stretch and agonist activation. We do not, however, view this $R_{stretch}$ as being solely related to a change in the muscle's position on the length-tension curve, since it was seen even at concentrations of agonists which did not generate any tone on their own (and therefore there would be no change in airway diameter / muscle length during the transition from *v* to *vi* in Fig. 1A and 1B.

Discrepancies between stretch applied to intact airways *versus* isolated ASM bundles have been noted, where in intact airways, stretch promoted increased muscle contractility and the opposite effect is seen in ASM bundles (Khangure et al., 2004;Noble et al., 2004). These discrepancies may be species-related and/or attributed to different properties of different regions in the airway tree, where $R_{stretch}$ may be a more significant phenomenon in small resistance airways compared to larger airways. A possible explanation for the stretch-induced contraction we observe is that following priming of the contractile apparatus with an agonist, stretch caused fluidization of the cytoskeleton, and during redevelopment of force, the contractile apparatus was able to regenerate force above and beyond pre-stretch levels. However, further experiments suggest the possibility that the $R_{stretch}$ phenomenon we observe in bovine bronchial segments may possess a neurogenic component.

DI-induced bronchoconstriction is abnormal in humans, given that it is only seen in moderate to severe asthmatics. Our bovine bronchial segments were not inflamed nor exhibited spontaneous tone, and did not manifest a stretch-induced contraction until they were pretreated with a contractile agonist (CCh or 5-HT), which we used to mimic the increased ASM tone seen

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in asthmatic airways. For our experimental setup, we chose to use a baseline transmural pressure of 5 cmH_2O, and maximal pressure pulse of 30 cmH_20, to mimic pressures in the human lung at FRC and TLC, respectively (Noble et al., 2007). In fact, we have demonstrated that pretreatment with CCh or 5-HT, at concentrations which produce relatively little change in basal tension, can produce a fundamental change in ASM biophysical properties and elicit a prolonged $R_{stretch}$. Given that $R_{stretch}$ is only seen in the presence of an agonist, it would appear to be non-myogenic in nature. Others have also demonstrated an $R_{stretch}$ in ASM that required pretreatment with a pharmacological agent to prime the contractile apparatus, such as tetraethylammonium chloride, or a cholinergic agonist (Stephens et al., 1975;Thulesius and Mustafa, 1994). The authors of these studies interpreted this phenomenon as a functional transformation of multiunit-smooth muscle into a single-unit, mediated by a contractile agonist. Although others (Fredberg et al. 1997) have observed a stretch-induced relaxation in bovine tracheal strips in the presence of an agonist (Ach), this was done using oscillatory stretches. The authors concluded that these tidal changes in length can cause an excess rate of detachment which is faster than the rate of attachment, thus causing a net decrease in ASM force production. In our setup, the airway stretch is static: as such the myosin and actin interactions should have been able to return to a latch state. Thus, the discrepancies between our data and those presented by Fredberg et al. appear to be due to differences in experimental protocols.

Mechanotransduction is sometimes mediated in part through activation of sensory neurons (Scotland et al., 2004). Also, neurogenic mechanisms can contribute to airway responsiveness (Joos et al., 2000;Widdicombe, 2003). Therefore, to investigate whether airway $R_{stretch,30}$ is also mediated by neuronal input, we treated the isolated airway segments with the sensory neurotoxin capsaicin (Geber et al., 2006). In this study, capsaicin-induced depletion of

sensory nerve endings abolished the $R_{stretch,30}$ which was unmasked by CCh, suggesting the involvement of sensory neurons. Surprisingly, we found this sensory neuronal component to be unaffected by TTX. TTX-resistant channels have previously been characterized on neurons controlling many different organ systems, including $Na_v1.8$ and Na_x which are expressed on sensory C-fibres and neurons in the peripheral nervous system with nerve endings in proximity of smooth muscle, respectively (Ogata and Ohishi, 2002).

Upon demonstrating a TTX-resistant sensory neuronal pathway involvement in $R_{stretch,30}$, we sought to characterize the neuronal pathway that mediates this response. Of the numerous neurotransmitters found within airway sensory nerve terminals, SP and NKA have been shown to contribute to bronchoconstriction in asthmatics. The receptors for these neurotransmitters, NK_1 and NK_2, respectively, have been well-characterized in ASM (Joos et al., 2000). Using the NK_1-receptor antagonist L-732,138, as well as the peptide NK_2-receptor antagonist, MEN 10376 and the non-peptide NK_2-receptor antagonist SR48968, we found no significant difference upon blockade of NK_1-receptors, whereas NK_2-receptor blockade significantly decreased contractile responses, thus affirming a central role for those receptors (and for NKA) in airway $R_{stretch,30}$. These results are supported by a recent study that showed an NK_2-selectivity pertaining to bronchial hyperreactivity and suggested an importance for capsaicin-sensitive nerves in bronchoconstriction in mice (Elekes et al., 2007). Another study found that NK_2 receptors played a predominant role in a guinea-pig model of mechanically-induced bronchoconstriction (Corboz et al., 2008). Conversely, protease-activated receptor-2 (PAR-2) mediated, TTX- and capsaicin-sensitive neurons in murine small intestine did not reveal differences in NK_1 vs. NK_2 selectivity (Zhao and Shea-Donohue, 2003), as we observed, which could possibly be explained by species differences between bovine and murine or tissue differences between bronchi and small intestine.

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Given that mechanotransduction often involves stretch-sensitive ion channels, we also probed the effect of various cation channel blockers on $R_{stretch,30}$. On the one hand, a significant inhibitory effect of Gd^{3+} implicated a central role for non-selective cation channels in these contractions; it is as yet unclear if these are the same set of Gd^{3+}-sensitive channels which we have previously shown are activated by intracellular Ca^{2+} store depletion in bovine ASM cells (Helli et al., 2005). Nifedipine -- a dihydropyridine class of L-type Ca^{2+}-channel blocker -- was also tested, albeit originally as a negative control for Gd^{3+}. Surprisingly, nifedipine also abolished $R_{stretch,30}$. To determine whether this was a non-specific effect of nifedipine, we then employed two other structural classes of L-type Ca^{2+} channel blocker -- verapamil (a phenylalkylamine) and diltiazem (a benzothiazepine) -- and found these too abolished $R_{stretch,30}$. L-type Ca^{2+} channels have been well-characterized in ASM (Green et al., 1993;Janssen, 1997); however, the electrophysiological and pharmacological properties of those channels are not consistent with an involvement in agonist-evoked responses (Janssen, 2002). Nonetheless, our data clearly suggest that airway stretch-activated contractions may signal through a different pathway than agonist-evoked contractions due to their dependence on L-type Ca^{2+} channels.

In conclusion, our data suggest that airway $R_{stretch}$ may occur through a non-myogenic pathway (since pretreatment with a contractile agonist is required) and airway sensory C-fibres are involved in mediating $R_{stretch}$ in bronchial segments. Moreover, it appears that this mechanosensitivity is sensed by stretch-activated cation channels. Following an elevation in transmural pressure, we propose that stretch-activated cation channels located on C-fibres penetrating the airway wall are activated, resulting in the release of NKA from these nerve-endings. This NKA, in turn, binds to postjunctional NK_2 receptors located on the smooth muscle to mediate an airway stretch-activated contraction. These results highlight an alternative pathway

for potential therapeutic targeting in asthmatic patients where a bronchoconstrictory response to a DI may play a role in airway hyperresponsiveness.

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Footnotes

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Legends for Figures

Fig 1. Experimental protocols for this study. (A) Pressure recording during the various manipulations used in our experimental protocol; details are given in the Methods, Results, and Discussion. Response to a pressure pulse stretch (30 cm H_20) is indicated by "↑". Restoration of transmural pressure to 5 cmH_2O is indicated by "↓". $R_{stretch}$ was quantified as illustrated. Italicized labels refer to the cartoon drawings of airway cross-sections given in (B), summarizing (in a non-quantitative fashion) the changes in pressure (P), volume (V) and airway diameter (L) during the various steps in our experimental protocol.

Fig 2. Effects of CCh and 5-HT pretreatment on bronchial responsiveness to stretch. Experiments were performed under isovolumic conditions. Agonists were added to the bath 10 min prior to the experimental protocol. (A) An instantaneous transmural stretch to 30 cmH_2O (from a baseline of 5 cm H_2O) elicited a contraction in airways pretreated with a contractile agonist (10^{-8}M CCh or 10^{-7}M 5-HT) but not in unpretreated tissues. Mean magnitudes of $R_{stretch}$ evoked by transmural pressures of 10-30 cmH_20 in the absence or presence of 10^{-8}M CCh (B) or 10^{-7}M 5-HT (C). n = 6 for both. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Fig 3. Relationship between contractile agonist concentration and $R_{stretch}$. Experiments were performed using the protocol illustrated in Figure 1B; agonists were added to the bath 10 min prior to the experimental protocol. Individual $R_{stretch,30}$ were measured at various concentrations of CCh (A) (n = 5) or 5-HT (C) (n = 6). Agonist-induced tone was measured under isovolumic

conditions by pretreatment with increasing concentrations of (B) CCh or (D) 5-HT. *n* = 6 for both.

Fig 4. Effects of mechanically-gated cation channel blockade on $R_{stretch,30}$. Mean values of $R_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with Gd^{3+} (10^{-3}M). *n* = 6. *, $p < 0.05$

Fig 5. Effect of L-type Ca^{2+} channel blockers on $R_{stretch,30}$. Mean values of $R_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with nifedipine ($2x10^{-6}$M; *n* = 6), verapamil (10^{-5}M; *n* = 6) or diltiazem (10^{-5}M; *n* = 6). ***, $p < 0.001$

Fig 6. Effect of neurotoxin treatment (capsaicin and TTX) on $R_{stretch,30}$. Mean values of $R_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with capsaicin (10^{-5}M; *n* = 6) or TTX (10^{-6}M; *n* = 6). **, $p < 0.01$

Fig 7. Effects of NK_1- and NK_2- receptor blockade on $R_{stretch,30}$. Mean values of $R_{stretch,30}$ measured before (open bars) and during (closed bars) treatment with the NK_1 receptor antagonist L-732,138 (10^{-5}M; *n* = 6) or the NK2 receptor antagonists MEN 10376 (10^{-5}M; *n* = 6) or SR48968 (10^{-6}M; *n* = 6). **, $p < 0.01$; ***, $p < 0.001$

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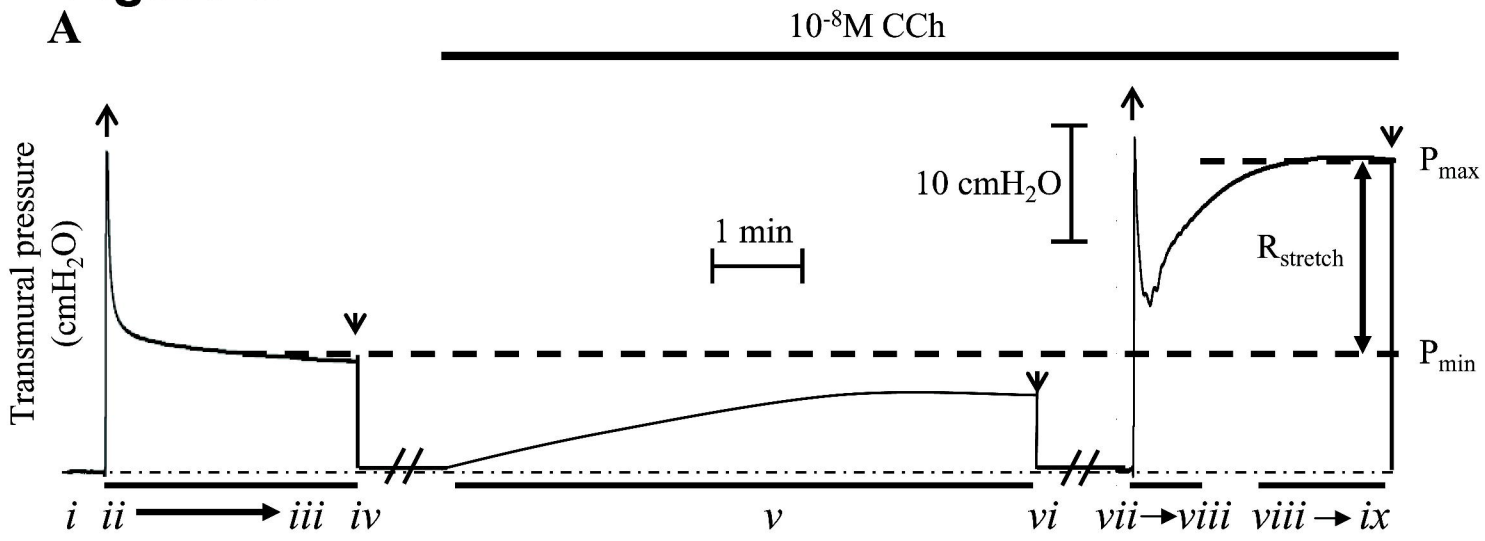
Figure 1.



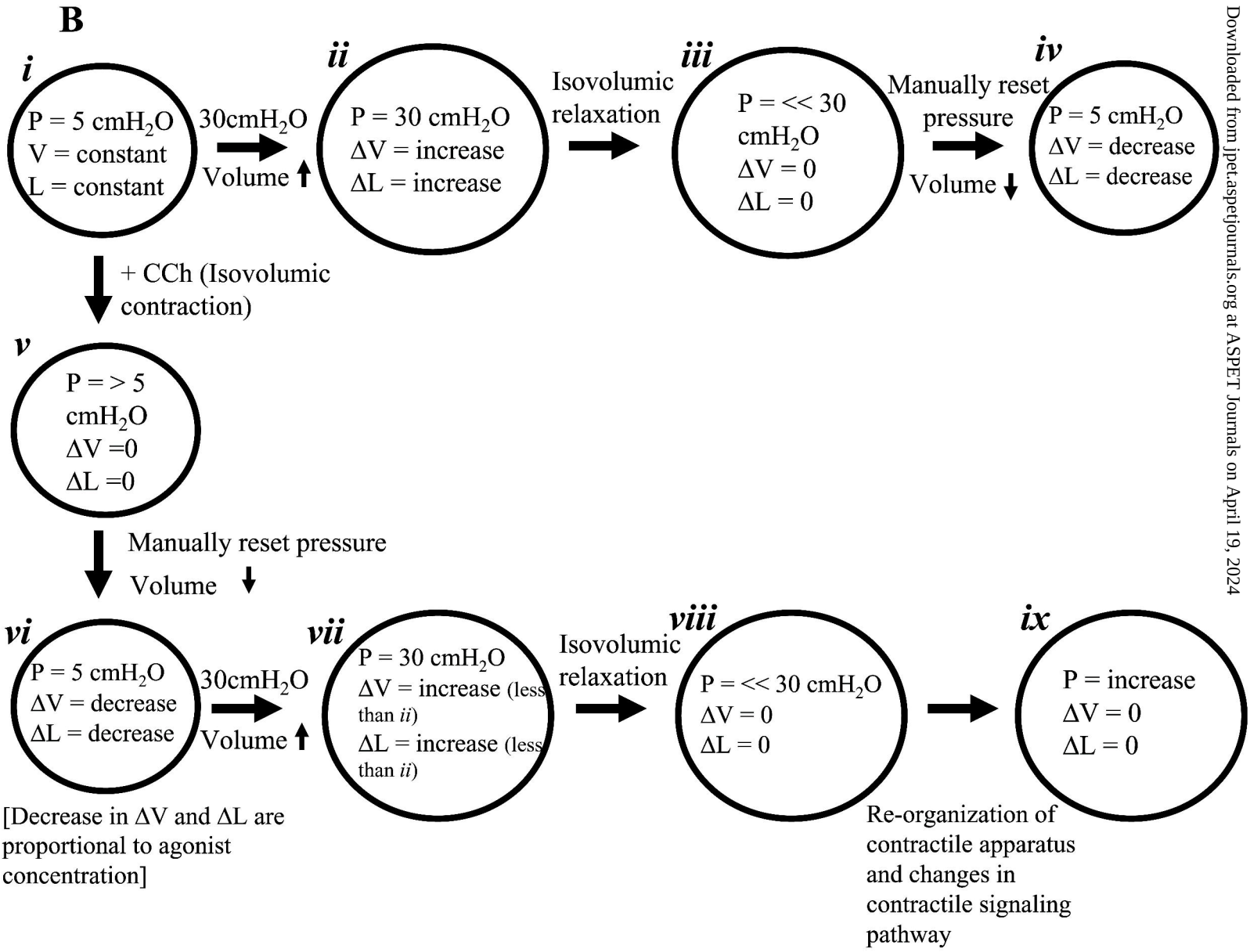


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Figure 2.

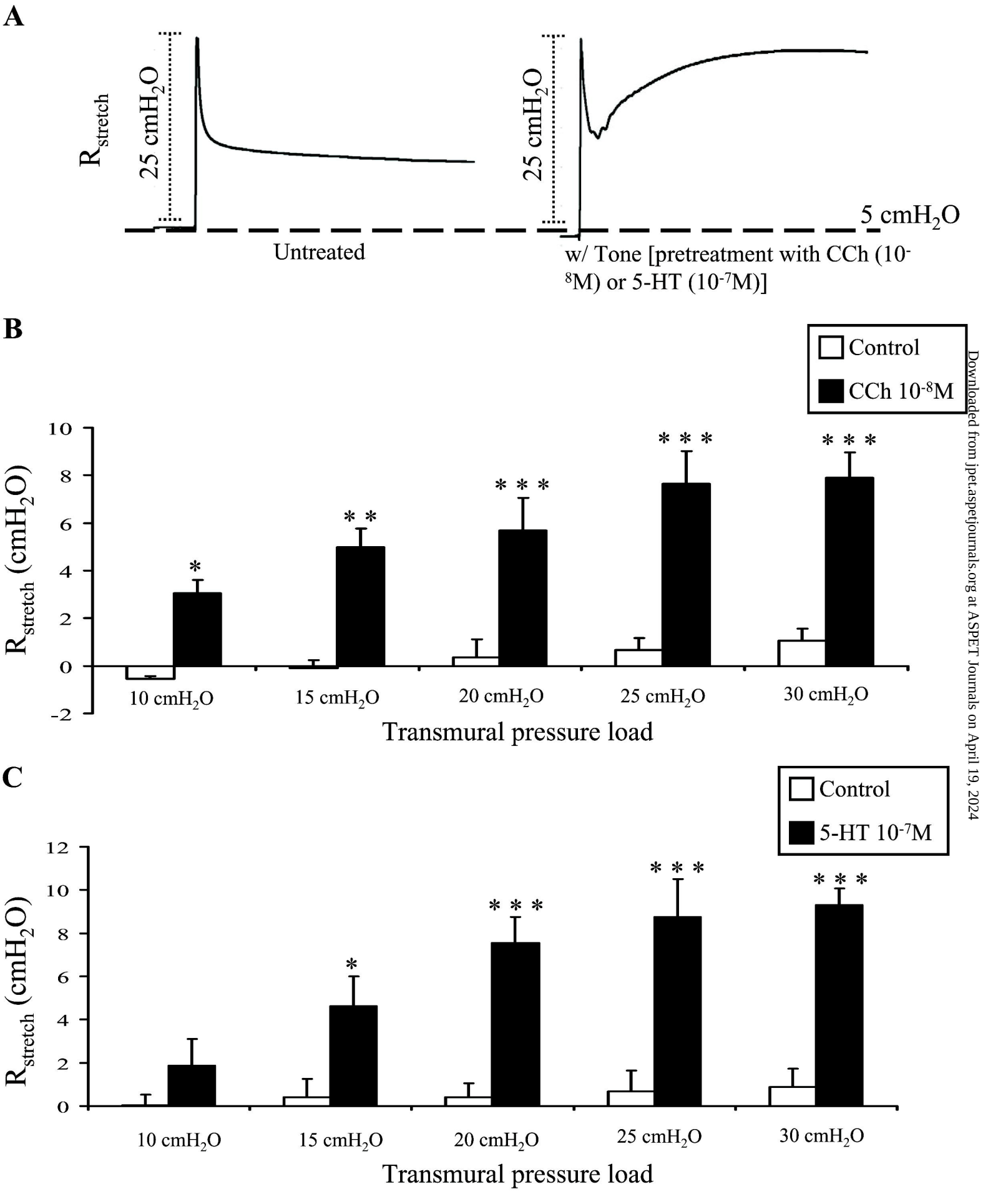

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Figure 3.

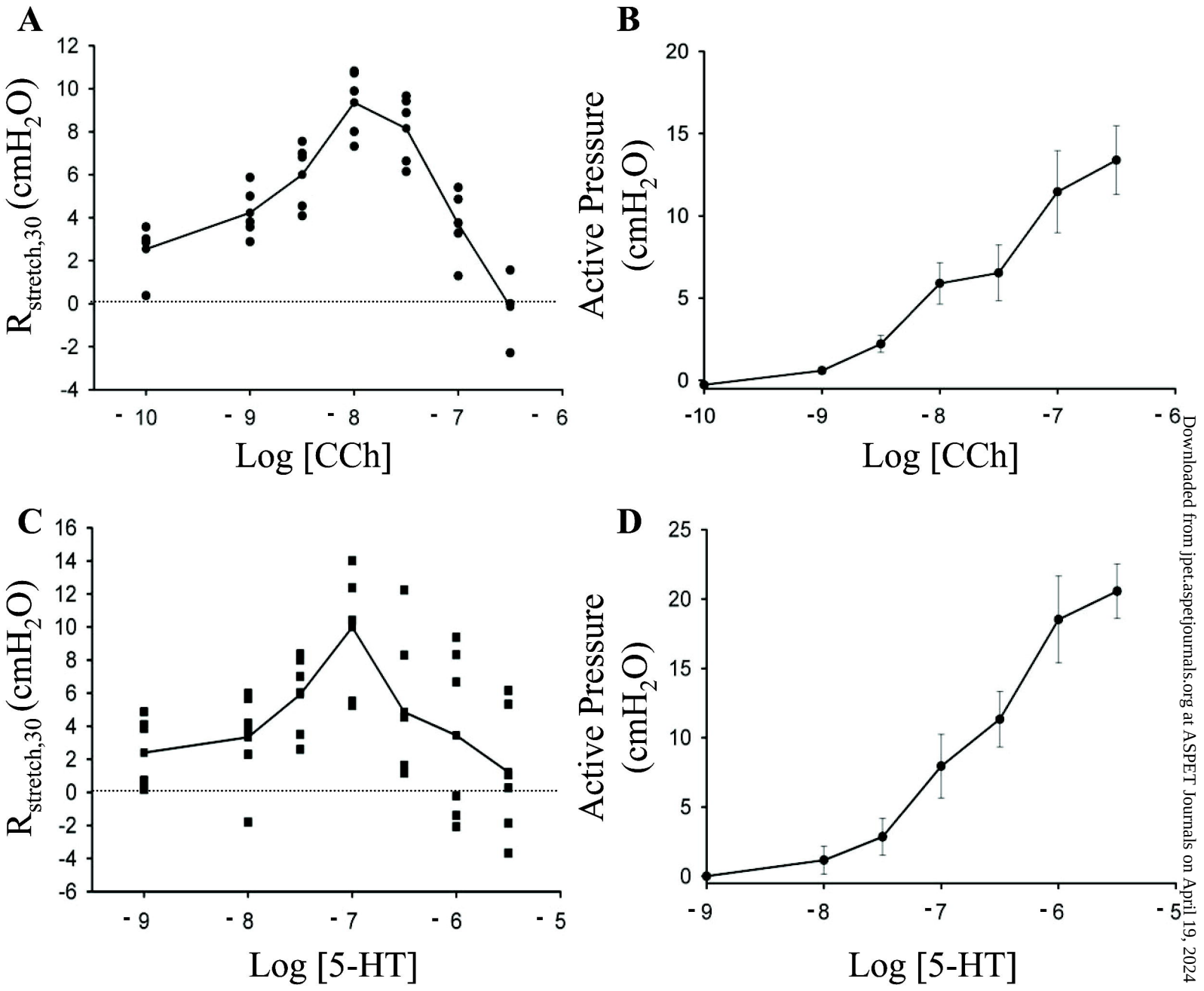

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Figure 4.

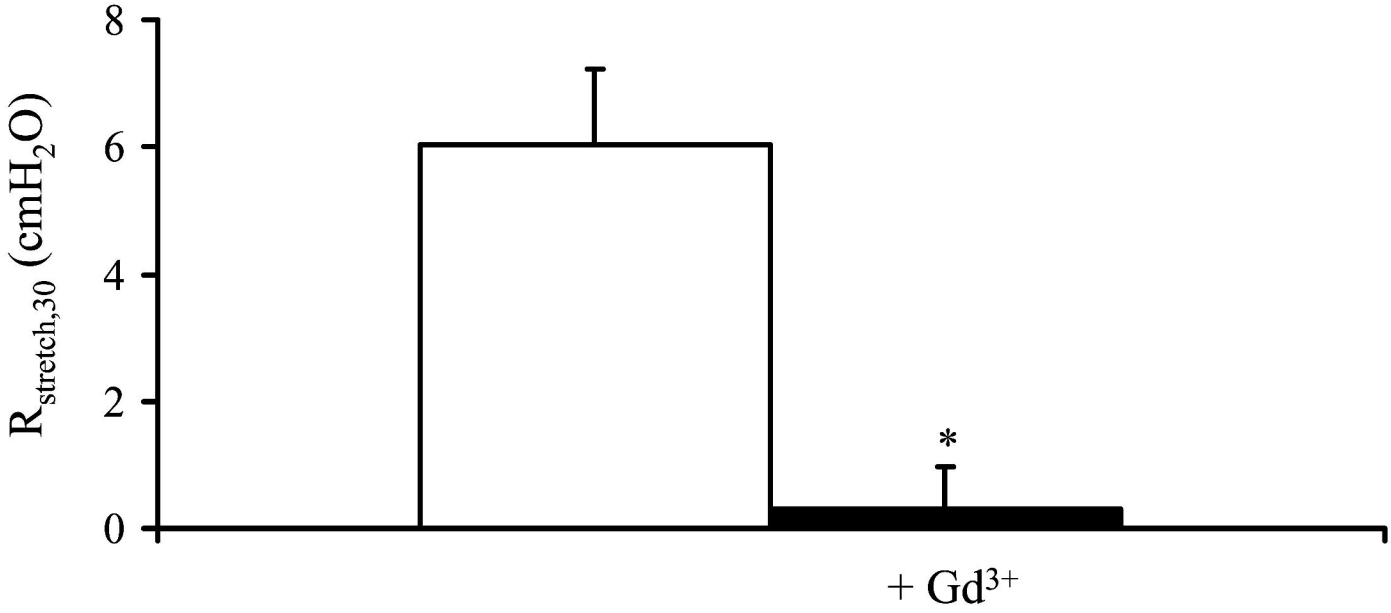

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Figure 5.

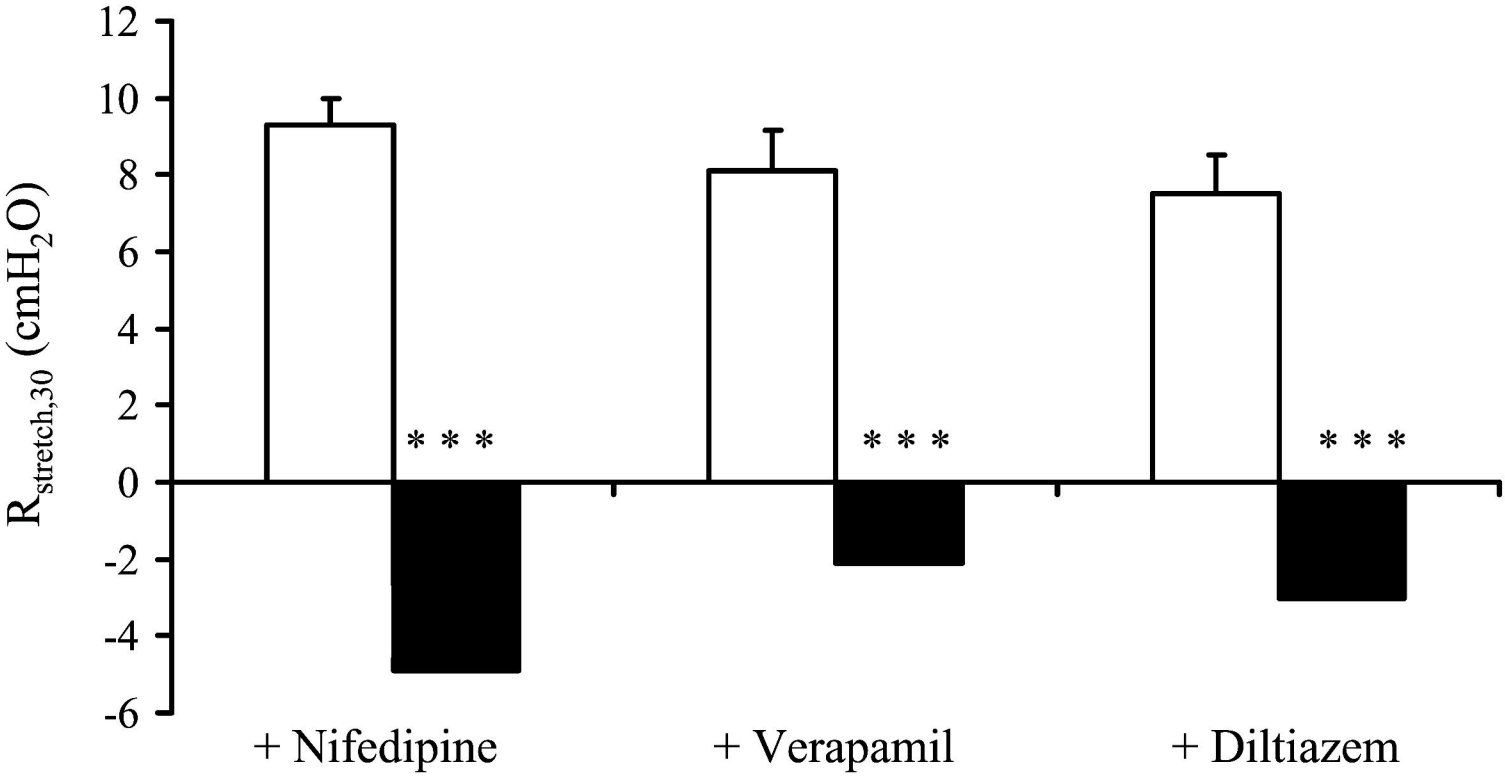

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Figure 6.

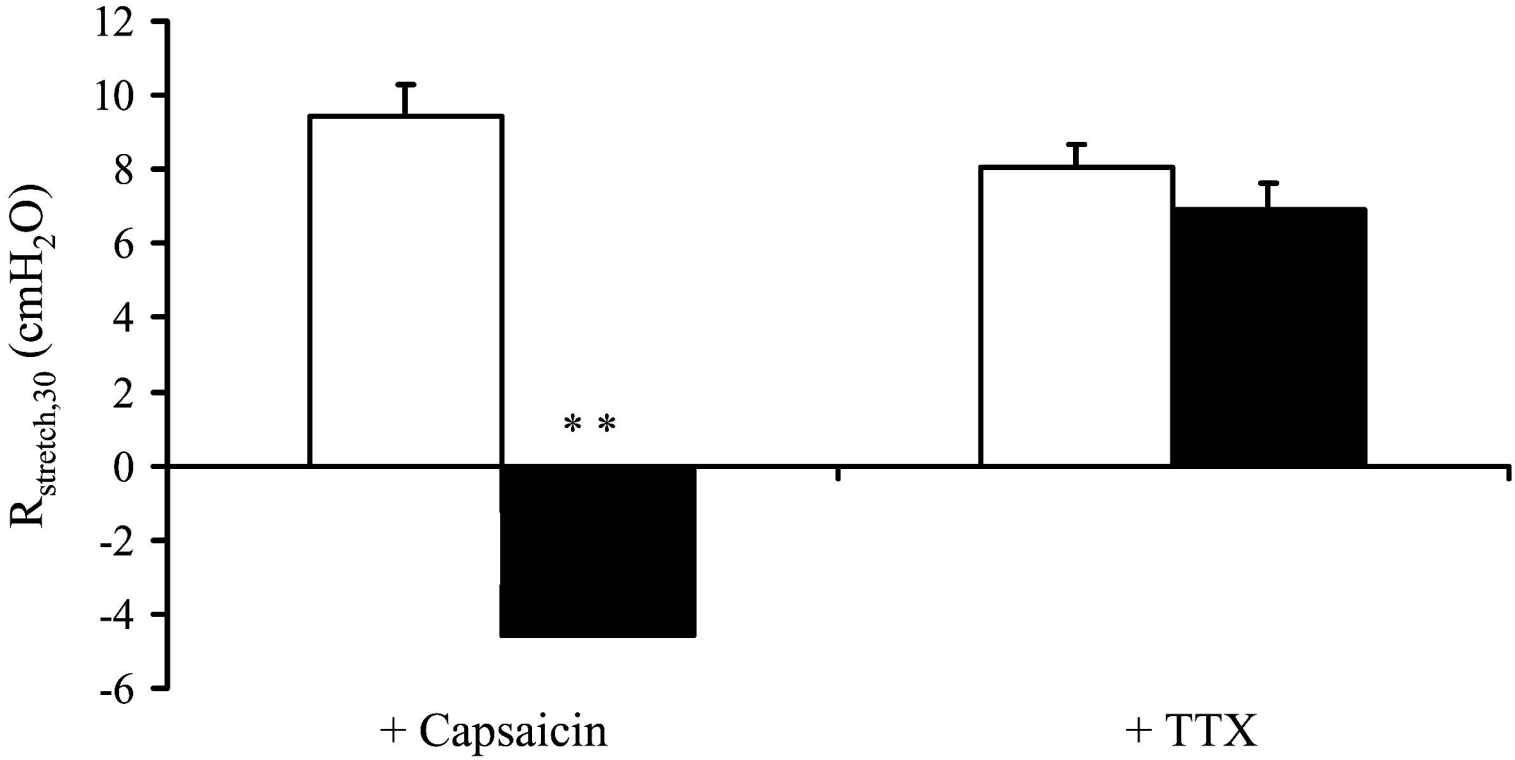

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Figure 7.