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Oxidative stress as activators of sensory nerves for cough

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Abstract

Excessive activation of the cough reflex is a major clinical problem in respiratory diseases. The cough reflex is triggered by activation of nociceptive sensory nerve terminals innervating the airways by noxious stimuli. Oxidative stress is a noxious stimuli associated with inhalation of pollutants and inflammatory airway disease. Here, we discuss recent findings that oxidative stress, in particular downstream of mitochondrial dysfunction, evokes increased electrical activity in airway nociceptive sensory nerves. Mechanisms include activation of transient receptor potential (TRP) channels and protein kinase C. Such mechanisms may contribute to excessive cough reflexes in respiratory diseases.

Keywords

Cough; Nociception; Reactive Oxygen Species; Mitochondria; TRP channel; Protein kinase C

Introduction

The cough reflex serves to protect the airways of mammals from noxious substances/events. Guinea pigs and larger mammals have been shown to cough in response to multiple airway stimuli, including mechanical perturbation, acid, water and irritants [1]. Electrophysiological recordings have demonstrated that the cough reflex is dependent on the activation of sensory afferent nerves innervating the airways, and that these nerves are specifically stimulated by tussive stimuli [2, 3]. Although mice and rats do not cough, they also have similar afferent innervation of the airways.

Despite the fact that the cough reflex is in general protective, chronic cough or cough hypersensitivity is a major clinical issue. It is thought that excessive activity in cough afferents contributes to an excessive cough reflex and there has been considerable effort made in elucidating the underlying mechanisms. In particular we have focused on oxidative stress as a likely contributor to cough hypersensitivity. As we shall discuss in more detail

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below, the lung is exposed to many oxidative and/or electrophilic agents and, furthermore, the afferents themselves may even be the source of oxidative stress during inflammation.

Afferents involved in cough

The afferent innervation of the airways is almost exclusively derived from vagal sensory nerves [2, 4, 5]. Sensory afferents are heterogeneous with respect to protein expression, structure, conduction velocity and function. At the most basic level, sensory afferents are either (1) low threshold mechanosensors involved in the homeostatic control of breathing or (2) afferents activated by noxious stimuli (termed 'nociceptors') involved in defense of the airways [6]. However, further complexity of phenotypes has been described. In particular, in guinea pigs and larger mammals the vagal ganglion is split into two separate structures: the nodose vagal ganglion and the jugular vagal ganglion. The phenotype of a given airway afferent is, in part, dependent its ganglionic origin [5, 7, 8]. In mice the ganglia are fused, but the distinct nodose and jugular phenotypes have been observed within airway afferent populations [9].

Overwhelming electrophysiological and behavioral studies demonstrate that cough can be elicited through two separate but interacting afferent pathways. Firstly, cough can be elicited in anesthetized animals by mechanical punctate stimulation, acid and nonisotonic solutions (e.g. water) applied to the larynx, trachea and main bronchi [3]. These responses are dependent on the activation of nodose Aδ fibers, which are sensitive to punctate force, acid and non-isotonic solutions [2, 3]. Secondly, cough can be elicited in conscious animals by a vast range of irritants and inflammatory mediators, such as capsaicin, bradykinin, acid, acrolein, allyl isothiocyanate, cinnamaldehyde and cigarette smoke [3, 10–16]. These responses are dependent on the activation of jugular C fibers, which are polymodal sensors of noxious stimuli due to their expression of multiple ion channels and receptors (see below). Cough evoked by activation of C fibers is blocked by anesthesia. Nevertheless, C fiber activity increases the sensitivity of Aδ fiber cough under anesthesia due to pathway interactions within the brainstem [17].

Oxidative stress is a noxious stimulus

Oxidative stress is a term that defines an aberrant redox state in which phospholipids, proteins and nucleic acids are oxidized by reactive oxygen species (ROS) causing major cellular dysfunction [18]. Oxidation and autooxidation of phospholipids can also lead to further damage via the actions of electrophilic products of peroxidation and nitration (e.g. alpha-beta-unsaturated carbonyl groups such as 4-hydroxynonenal) [19].

Oxidative stress in the airways can arise from 2 distinct sources: inhalation of electrophilic irritants and endogenous inflammation. Air pollution from multiple sources is replete with oxidative/electrophilic agents such as ozone, acrolein, chlorine, aldehydes and isocyanates. The initial step in endogenous oxidative stress is the formation of the ROS superoxide, which can then converted into hydrogen peroxide and the hydroxyl radical depending on conditions [18]. Endogenous superoxide is mainly derived from NADPH oxidase, xanthine oxidase and inefficiencies in mitochondrial electron transfer within mitochondria. Under resting conditions NADPH oxidase and xanthine oxidase are inactive and only a tiny amount

of superoxide is produced from mitochondria. However, with inflammatory conditions superoxide production dramatically increases. In particular, NADPH oxidase is activated in infiltrating leukocytes such as neutrophils and eosinophils as part of phagocytosis. In addition, inflammatory pathways such as TNFα[20], neurotrophins via p75NTR [21], TGFβ[22] and Toll-like Receptors [23] have been shown to increase ROS production from mitochondria. Regardless of the source, oxidative stress is a major threat to airway cellular function and as such oxidative stress can be considered a noxious stimuli.

Activation of airway C fibers by oxidative stress and electrophilic irritants

The activation of sensory nerve terminals (i.e. action potential discharge) by irritants is dependent on ion fluxes through specific irritant-sensitive ion channels in the plasma membrane. Extensive efforts have been made to understand the ion channels involved in the activation of airway C fibers by noxious stimuli. For example, the canonical noxious stimulus capsaicin [24], the pungent ingredient in chili peppers, activates airway C fibers through the gating of transient receptor potential (TRP) vanilloid 1 (V1) [25, 26]. TRPV1 expression is largely restricted to nociceptive afferents, and inhibition or genetic ablation of TRPV1 renders nerves insensitive to capsaicin. As such capsaicin-induced cough in conscious guinea pigs is abolished by TRPV1 inhibitors [27, 28].

Another member of the TRP superfamily, TRP ankyrin 1 (A1), is also selectively expressed in nociceptive sensory nerves [29]. TRPA1 was shown to be activated by multiple irritants such as the isothiocyanates, cinnamaldehyde and acrolein and ablation of TRPA1 largely abolished nociceptor activation by these irritants [29–31]. The similarities in electrophilicity, not structure, of TRPA1 activators suggested that covalent modification of TRPA1 thiols was critical for its activation. Evidence for this unusual activation mechanism was demonstrated in studies of mutant channels with select point mutations of N-terminal cysteines that had greatly reduced sensitivity to electrophiles [32, 33].

In the mouse airways, TRPA1 is expressed on the majority of slowly-conducting nociceptive C fibers [34]. These fibers are robustly activated by cinnamaldehyde and allyl isothiocyanate (AITC), and such responses are inhibited by TRPA1 inhibition or genetic ablation [34–36]. Furthermore approximately 30–40% of dissociated vagal neurons are activated by TRPA1 agonists (again abolished in TRPA1 knockout neurons) [34, 35, 37]. In vivo inhalation of AITC evoked TRPA1-dependent reflex bradypnea in mice, identical to the defensive reflexes evoked by capsaicin [38]. In the guinea pig, TRPA1 agonists activate dissociated jugular nociceptive neurons [39] and jugular C fibers innervating the airways [16]. Consistent with the reported role of jugular C fibers in cough reflexes, inhalation of AITC, cinnamaldehyde and acrolein causes cough in conscious guinea pigs in a TRPA1 inhibitor-sensitive manner [12, 13, 16]. TRPA1 agonists, like capsaicin, failed to activate nodose Aδ fibers innervating the guinea pig trachea [16].

Given that TRPA1 activation correlated with electrophilic reactivity rather than structure, we investigated the potential for other electrophiles, present in air pollution or endogenously as part of the inflammatory response, to activate TRPA1 on airway nociceptors.

We focused on the exogenous irritants toluene diisocyanate (TDI, product of the manufacture of polyurethane) and ozone (formed by the reaction of exhaust pollutants in sunlight), both of which are associated with respiratory morbidity including cough, dyspnea and chest tightness [40, 41]. Both TDI and ozone activated human TRPA1 channels when expressed in HEK293 cells [38, 42]. Furthermore, TDI and ozone activated a subset of vagal nociceptive neurons in wild-type mice but not TRPA1 knockout mice. In recordings of airway C fibers, ozone only activated TRPA1-sensitive afferents and these responses were inhibited by ruthenium red (a TRP inhibitor). Like AITC, TDI evoked bradypnea with an associated increase in 'time of break' in wild-type mice but not TRPA1 knockout mice.

As mentioned above, inflammation is associated with oxidative stress and the production of numerous electrophiles including 4-hydroxynonenal (4HNE), 4-oxononenal (4ONE), prostaglandin A_2, 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2 and 9-nitrooeate [43–45]. Consistent with their electrophilicity, all these compounds activated human TRPA1, unlike their nonreactive analogs (e.g. prostaglandins B_2 and D_2 and oleic acid) [35, 37, 46, 47]. 4ONE also activated human TRPV1, albeit at higher concentrations [35]. 4HNE, prostaglandin A_2, 15-deoxy- $\Delta^{12,14}$-prostaglandin J_2 and 9-nitrooeate selectively activated dissociated nociceptive neurons, which was abolished by TRPA1 inhibition and/or genetic ablation. 4ONE activated vagal dissociated neurons via a combination of TRPA1 and TRPV1. In recordings of airway C fibers, both 4ONE and 9-nitrooeate caused robust action potential discharge. Other groups identified hydrogen peroxide as an activator of TRPA1 [46, 48], and we have recently shown that 1mM H_2O_2 selectively activates AITCsensitive airway C fibers, but fails to activate AITC-insensitive fibers [49]. Higher concentrations of H_2O_2 (120mM) activate bronchopulmonary C-fibers in the rat via a combination of TRPA1 and P2X channels [50].

Afferent terminal mitochondria as a potential source of ROS

The peripheral nerve terminals of vagal airway sensory fibers are densely packed with mitochondria [51–53]. Mitochondria can also be observed in axonal terminals of vagal neurons in long-term culture [54]. In order to further understand the functional effect of increased mitochondrial ROS on airway afferent, we have used specific inhibitors of the mitochondrial electron transfer chain. Antimycin A selectively inhibits the Q_i site on complex III, resulting in superoxide formation and the subsequent production of numerous ROS, mitochondrial membrane depolarization and a decrease in ATP production [55–58]. Whereas Inhibition of the Q_o site on complex III by myxothiazol causes mitochondrial membrane potential depolarization and a decrease in ATP production but only very mild superoxide production [55, 57, 58]. Furthermore, inhibition of Q_o substantially reduces subsequent superoxide production evoked by inhibition of the Q_i site (antimycin A) [55, 57, 58]. Finally, oligomycin inhibits complex V (also known as ATP synthase) and thus prevents ATP production by the oxidative phosphorylation machinery [59, 60]. Inhibition of complex V also evokes a mild hyperpolarization of the mitochondrial membrane potential but has little effect on superoxide production in most systems.

Surprisingly, ATP production from these terminal mitochondria does not seem to be required for the maintenance of electrical gradients (glycolysis is sufficient). Inhibition of

mitochondrial ATP production failed to cause significant reductions in the activity of either peripheral afferents or dissociated vagal neurons of the course of more than 1 hour recordings [36, 49, 61]. Nevertheless, we expect that chronic exposure to these mitochondrial inhibitors may lead to loss of terminal function. Given the density of mitochondria in airway terminals and their close proximity with the transductive machinery of the terminal, we hypothesized that mitochondrial ROS may modulate airway afferent activity.

Mitochondrial ROS activate airway nociceptors via TRP channels

Antimycin A (20 μM), which evokes ROS from mitochondrial complex III, evoked action potential discharge from nociceptive C fiber terminals innervating the mouse airways [36]. Antimycin A-induced nociceptive C fiber activation was significantly greater in nociceptors that expressed TRPA1 compared to nociceptors that did not express TRPA1. Antimycin A failed to activate non-nociceptive fibers. Consistent with the sensitivity of TRPA1 to ROS, the antimycin A-induced action potential discharge was reduced by the TRPA1 antagonist HC-030031 (30 μM), although inhibition of TRPV1 with iodoresiniferatoxin (1 μM) further reduced C-fiber activation. Similar responses were observed in Fura 2AM Ca2+ imaging studies of dissociated mouse vagal neurons. Antimycin A (20 μM) evoked Ca2+ influx in nociceptive neurons that was inhibited by approximately 50% either by genetic ablation of TRPA1 or by HC-030031 (30 μM). The residual Antimycin A-induced Ca2+ responses were sensitive to TRPV1 inhibition. Such data suggested that TRPA1 is the primary mechanism by which mitochondrial ROS activate airway nociceptors, although TRPV1 may also contribute.

We investigated the direct effect of mitochondrial ROS on TRPA1 and TRPV1 function in HEK293 cells [36]. Antimycin A induced concentration-dependent activation of both human TRPA1 and human TRPV1 but failed to activate non-transfected cells. TRPA1 was more sensitive to mitochondrial ROS. Antimycin A-induced TRPA1 activation was prevented by pretreatment with myxothiazol, confirming the role of ROS produced from complex III. Furthermore a combination of tempol (superoxide dismutase mimetic) and MnTMPyP (super oxide dismutase and catalase mimetic) reduced antimycin A-induced TRPA1 activation by 75%. ROS such as H_2O_2 could activate TRPA1 either directly [46] or indirectly through their well characterized production of electrophilic lipid peroxidation products such as 4HNE [35]. Reducing agents such as dithiothreitol can prevent/reverse ROS-induced oxidation of cysteines, but have no effect on the Michael reaction underlying covalent modification of cysteines by electrophiles [46]. We found that dithiothreitol effectively reduced Antimycin A-induced TRPA1 activation but had no effect on AITC-induced TRPA1 activation, suggesting that mitochondrial ROS themselves directly activated TRPA1.

Mitochondrial ROS increase airway nociceptor excitability via PKC

Antimycin A (20 μM) had a profound effect on the excitability of nociceptive C fibers innervating the mouse airways [49]. Antimycin A decreased the threshold sensitivity for mechanical punctate stimulation by 50% and increased the action potential firing elicited by

a $P2X_{2/3}$ agonist (α, β methylene ATP, 30 μM) to 270% of control. Antimycin A had no effect on the excitability of non-nociceptive airway afferents. Antimycin A-induced nociceptor hyperexcitability was independent of TRPA1 expression and was not reduced by either TRPV1 inhibition (iodoresiniferatoxin, 1 μM) or in TRPV1 knockout afferents. Neither myxothiazol nor oligomycin induced nociceptor hyperexcitability, indicating that antimycin A-induced responses were dependent on mitochondrial ROS production and not dependent on the inhibition of mitochondrial ATP production. Consistent with this, H_2O_2 (1mM) also increased nociceptor excitability (to 207% of control). Antimycin A-induced hyperexcitability was inhibited by dithiothreitol (membrane-permeable reducing agent) and by N-acetyl cysteine (membrane-permeable antioxidant) but not by glutathione (membrane-impermeable antioxidant), suggesting the involvement of intraneuronal ROS. Furthermore, antimycin A-induced hyperexcitability was prevented by myxothiazol pretreatment.

Previous studies of vagal nociceptors have shown that protein kinase C (PKC) is a powerful regulator of excitability [62–66]. Interestingly, many PKC isoforms can be activated by cellular oxidants (e.g. superoxide and H_2O_2) independently of either Ca^{2+} or diacylglycerol [67–69]. We therefore hypothesized that mitochondrial ROS induce airway nociceptor hyperexcitability via activation of protein kinase C. Consistent with this hypothesis antimycin A-induced hyperexcitability was reduced by approximately 80% by the PKC inhibitor bisindolylmaleimide (BIM) I, but was unaffected by the inactive analog BIM V [49]. Under resting conditions PKC is a cytosolic protein, which will translocate to the plasma membrane once activated. Using an antibody that binds all PKC isoforms, we found that antimycin A caused PKC translocation to the plasma membrane of dissociated vagal neurons. Antimycin A-induced PKC translocation was abolished by the pretreatment of the neurons with a combination of tempol and MnTMPyP, indicating a critical role of mitochondrial ROS in these responses. Furthermore, H_2O_2 also evoked PKC translocation.

At present, there are two major gaps in our understanding of mitochondrial ROS-induced hyperexcitability. Firstly, we have yet to determine which PKC isoform is involved. There are 15 isoforms of PKC and, based upon our preliminary RT-PCR studies, almost all are present in the vagal ganglia. Secondly, we do not yet understand how PKC activation leads to hyperexcitability. Activated PKC isoforms are capable of modifying neuronal excitability via the phosphorylation of multiple cellular targets such as ligand-gated ion channels, voltage-gated ion channels, leak channels and transporters. Nevertheless, it is important to note that non-nociceptive airway afferents were unaffected by antimycin A, and thus the putative mechanism must be selectively expressed only in the nociceptive population.

Conclusions

Oxidative stress, either in the form of ROS or electrophilic lipid product of peroxidation, causes significant increases in the activity of nociceptive neurons. Oxidative stress causes the activation of nociceptors via TRPA1 and increases the excitability of nociceptors via PKC. Nerve terminals of afferents innervating the airways are densely packed with mitochondria. ATP production from nerve terminal mitochondria is not required for continued electrical excitability. Given that multiple inflammatory pathways have been shown to stimulate mitochondrial ROS production we hypothesize that sensory terminal

mitochondria function as an integrated transduction mechanism that converts inflammatory signaling into intraneuronal ROS signaling capable of potent modulation of electrical activity. Presently, our studies have only used direct modulators of the mitochondria electron transfer chain. Further studies are needed to determine the role of mitochondrial ROS signaling in aberrant airway nociceptor function in inflammation. It is possible that these pathways contribute to chronic cough and cough hypersensitivity in airway disease.

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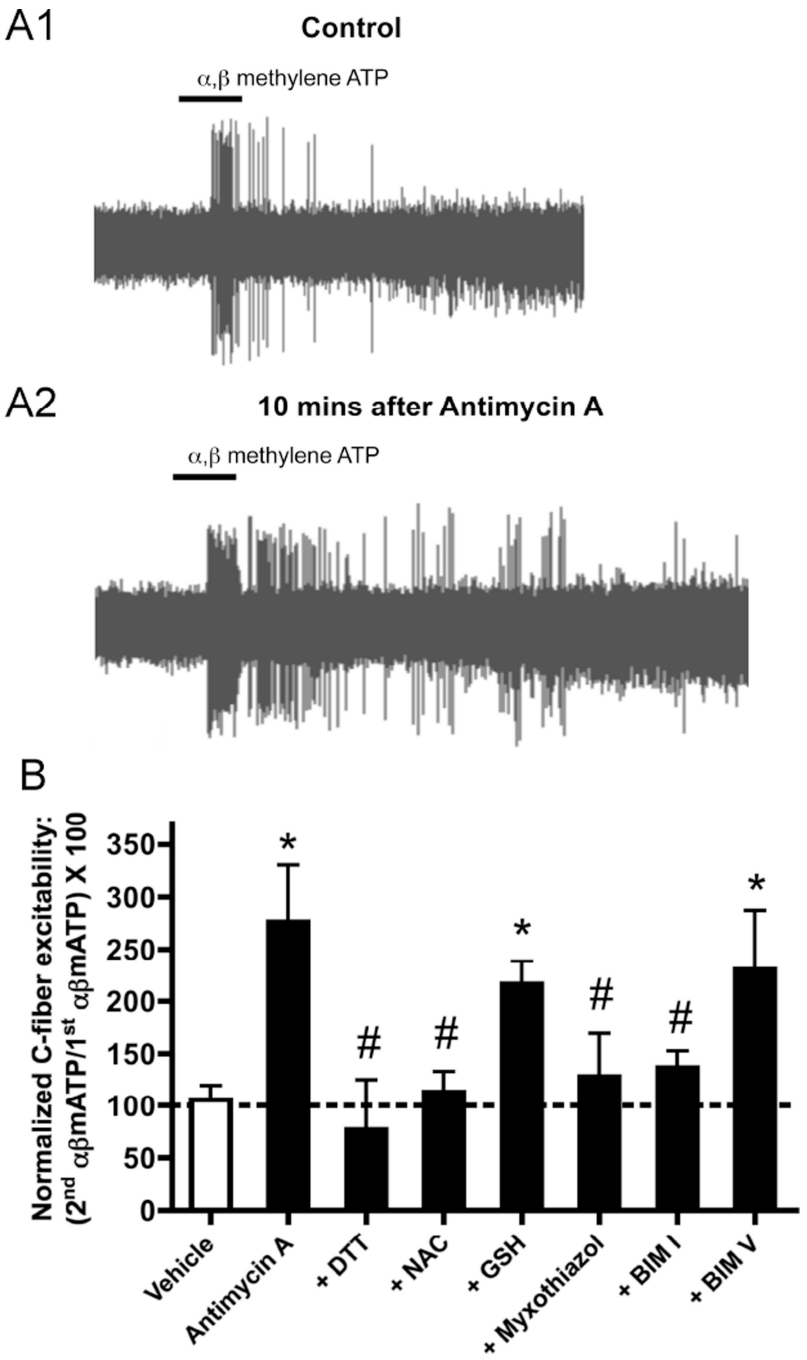

Fig. 1. Antimycin A activates nociceptive bronchopulmonary C-fibers via TRPA1 and TRPV1

A, representative trace showing action potential discharge to antimycin A (20 μM, blocked line denotes 10s application) in an individual TRPA1-expressing bronchopulmonary C-fiber. B, Mean ± SEM peak action potential discharge from individual bronchopulmonary C-fibers in response to antimycin A, grouped according to their response to selective TRPA1 agonists (data not shown). Data in all columns only includes *nociceptive* wild-type, defined by conduction velocity and sensitivity to capsaicin (1 μM) (data not shown). *Left*, responses in control wild-type TRPA1-expressing fibers (black column) are compared with responses

in the presence of 30 μM HC-030031 (gray column) and 1 mM GSH (hatched column). *Right*, responses in control wild-type fibers not expressing TRPA1 (black column) are compared with responses in the presence of 1 μM I-RTX. * Significant reduction compared to control ($p<0.05$). Adapted from [36].

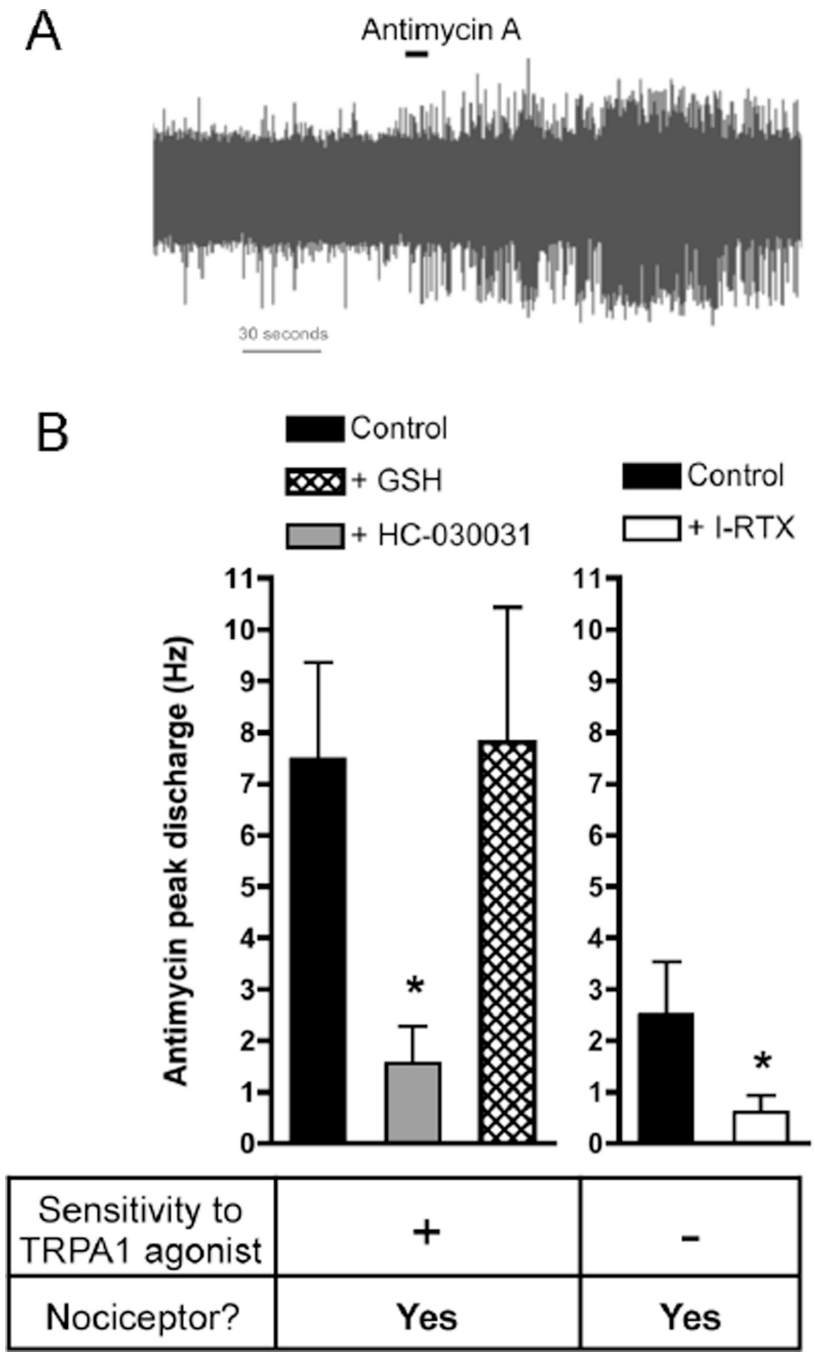

Fig. 2. Antimycin A increases nociceptive bronchopulmonary C-fibers excitability

A, representative traces of action potential discharge evoked by 10s challenge with α,β mATP ($P2X_{2/3}$ agonist, 30 μM) in a nociceptive bronchopulmonary Cfiber before (control, A1) and 10 minutes after treatment with antimycin A (20 μM, A2). B, mean ± SEM response to 2nd application of α, β mATP (30 μM) normalized to response to 1st application of α, β mATP prior to either vehicle (white bar) or antimycin A (20 μM, black bars) in nociceptive C-fibers. The roles of ROS and PKC in hyperexcitability to α, β mATP were determined using pretreatment with DTT (1mM), NAC (1mM), GSH (1mM), myxothiazol

(500 nM), BIM I (1 μM) or BIM V (1 μM). * Significant increase in 2nd α, β mATP-induced responses after antimycin A compared to vehicle ($p<0.05$). # Significant reduction in antimycin A-induced hyperexcitability to α, β mATP ($p<0.05$). Adapted from [49].